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Prevention of c-Jun/activator protein-1 activation and microsomal epoxide hydrolase induction in the rat liver by cysteine during protein–calorie malnutrition

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Abstract

Protein–calorie malnutrition (PCM), a major global health problem, arises during protein and/or energy deficit due to disease and nutritional inadequacy. To date, cellular adaptive responses and gene expression associated with PCM remain poorly understood. In view of the primary role of the liver in energy conversion, the present study was designed to investigate changes in hepatic morphology and molecular alterations during PCM. PCM caused marked decreases in the cytoplasmic eosinophilic content and nuclear shrinkage in the hepatocytes with a decrease in glutathione content. The nuclear activator protein-1 (AP-1) complex was activated in the liver of PCM rats. AP-1-binding activity of nuclear extracts produced from PCM rats was reduced by the presence of anti-c-Jun antibody. Microsomal epoxide hydrolase (mEH), a phase II detoxifying enzyme, was 4-fold induced, with a 20-fold increase in the mRNA level during PCM. In contrast to the PCM-induced changes in hepatic morphology, PCM rats supplemented with cysteine showed an increase in the GSH level and well-preserved hepatic structures with mild fat degeneration. Cysteine supplementation inhibited the activation of AP-1 and the induction of mEH in PCM rats. These results provided evidence: (i) that PCM alters liver morphology with a decrease in the glutathione level; (ii) that cysteine may serve as a key element responsible for preserving hepatic morphology and maintaining the glutathione level; and (iii) that cysteine was active in preventing the activation of AP-1 and mEH induction in the liver during PCM.

Keywords: protein–calorie malnutrition; c-Jun; cysteine; methionine; microsomal epoxide hydrolase; AP-1

1. Introduction

PCM[†] arises when there is insufficient energy or protein available to meet metabolic demands due to disease and increased nutrient losses [1]. Protein deficiency is present if the content of dietary protein is inadequate. The rapidity of PCM development is determined by factors such as nutritional adequacy and underlying diseases and physiologic states [1]. Low contents of certain essential amino acid(s) including citrulline, cysteine, ornithine, taurine, and tyrosine may become a limiting factor for PCM development [2]. Interconversions in energy substrates occur in the liver, which plays a major role in adaptive responses during protein deficiency. Changes in basal metabolism and energy utilization have been studied as part of the compensatory responses functioning during protein and/or energy deficit [3]. However, the cellular adaptive responses and gene expression associated with PCM remain poorly understood. Because protein restriction may lead to oxidative stress [4,5], cellular redox homeostasis and associated gene regulation may be affected.

Studies from our laboratories have shown that PCM influences the pharmacokinetics and pharmacodynamics of therapeutic agents [6,7]. Previously, we showed that PCM suppresses the expression of cytochrome P450s [8]. The metabolic basis as well as pharmacokinetic profiles should be investigated for PCM patients taking medication therapy. Understanding the regulatory and molecular basis for the altered metabolic profile would be of assistance for improved pharmacotherapy for individuals with PCM.

mEH converts arene and alkene oxides to vicinal dihydrodiols. mEH may also play a role in transporting bile

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[†] *Abbreviations:* PCM, protein–calorie malnutrition; mEH, microsomal epoxide hydrolase; AP-1, activator protein-1; and NF-κB, nuclear factor-κB.

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acids in the liver [9]. The expression of mEH is greatly affected by reactive oxygen species produced from the metabolism of xenobiotics [10,11]. Irradiation with γ-rays up-regulates the expression of mEH [12]. We were interested in the possible up-regulation of *mEH* gene expression in the liver during PCM. The expression of mEH in conjunction with the activation of AP-1 (a transcriptional factor associated with the cellular redox state) was assessed in the present study.

Cysteine prevents toxicant-induced liver injury, apparently through the mechanism involving elevation of the intracellular GSH level [13]. Cysteinyl residues are required for certain DNA-binding proteins and transcriptional factors involved in signal transduction and redox regulation [14]. The cellular signal controlled by free sulfhydryl-containing molecules may affect the associated gene expression [14–16]. Hence, sulfur-containing amino acids such as cysteine and methionine may serve as a potential limiting factor for determining PCM-induced responses. The present study was designed to further determine the effects of cysteine on morphological and molecular changes in the liver during PCM.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (3000 mCi/mmol) and [γ-^{32}P]ATP (3000 mCi/mmol) were purchased from New England Nuclear. The formulated isocaloric diets containing 5% or 23% casein were supplied by Dong-A Pharmaceutical Co., as described previously [8]. Antibodies specific for c-Jun, JunB, JunD, c-Fos, FosB, and Fra-1 were purchased from Santa Cruz Biotechnology. Biotinylated goat anti-rabbit immunoglobulin G and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were obtained from Life Technologies. The random prime-labeling kit was purchased from Promega. GSH-400, a GSH assay kit, was purchased from Oxis International. Most of the reagents in the molecular studies were supplied from Sigma Chemical Co.

2.2. Animal treatment

Male Sprague–Dawley rats (150–190 g) were purchased from Charles River and maintained in a clean room at the Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University at a temperature between 20° and 23° with 12-hr light and dark cycles and relative humidity of 50%. Animals were caged under the supply of filtered pathogen-free air and water *ad lib*. Rats at 5 weeks of age were randomly assigned to two groups that were fed either the control diet containing 23% casein or the low protein diet containing 5% casein for 4 weeks. Food intake and body weight were recorded at least once a week [8]. Animals were gavaged with either cysteine or methionine at the daily dose of 500 mg/kg body weight (250 mg/kg body weight twice per day) for the last 7 days during 4 weeks of protein restriction. Cysteine was dissolved in an aqueous solution, whereas methionine was suspended in a 0.1% carboxymethylcellulose solution. The body weight of control and PCM rats was 285 ± 23 and 157 ± 11 g, respectively, whereas the liver weight of the animals was 12.0 ± 2.2 and 6.9 ± 1.2 g, respectively [8]. Supplementation of PCM rats with cysteine or methionine caused no changes in the liver or body weight. Each datum point consisted of six independent experiments.

2.3. Histopathology

The effects of PCM with or without cysteine/methionine supplementation on liver morphology were assessed by light microscopy. Livers were removed and fixed in 10% buffered formalin, processed, and embedded in paraffin. Sections 5 μm in thickness were stained with hematoxylin and eosin prior to examination. The samples were scored by a certified pathologist in a blinded fashion.

2.4. Hepatic GSH content

The GSH content was measured using a commercial kit according to the manufacturer's protocol (Oxis International). Briefly, the GSH-400 method was based on a chemical reaction which proceeded in two steps. The first step led to the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluromethyl-quinolinum methylsulfate and all mercaptans present in the sample. The second step included a β-elimination reaction under an alkaline condition. This reaction was mediated by 30% NaOH, which specifically transformed the substituted product (thioether) obtained with GSH into a chromophoric thione.

2.5. Gel retardation assay

Double-stranded DNA probes for the consensus sequences of AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used for gel shift analyses after end-labeling of the probes with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Nuclear extracts were obtained by a modification of a procedure published previously [17]. The reaction mixtures contained 2 μL of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/mL of poly dI-dC, and 50 mM Tris–Cl (pH 7.5), 10 μg of nuclear extracts, and sterile water in a total volume of 10 μL. The reaction mixtures were preincubated for 10 min. DNA-binding reactions were carried out at room temperature for 20 min after the addition of 1-μL probe (10^6 cpm). Specificity of binding was determined by competition experiments that were carried out by adding a 10- or 20-fold excess of an unlabeled AP-1, specific protein-1, or AP-2 oligonucleotide to the reaction mix-

ture before the DNA-binding reaction. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried, followed by autoradiography.

In some experiments, 10 μg of nuclear extracts was incubated with 10 μg of highly specific anti-c-Jun, anti-JunB, anti-JunD, anti-c-Fos, anti-FosB, or anti-Fra-1 antibodies (Santa Cruz Biotechnology) at room temperature for 1 hr, according to the method described previously [18–20]. For immunodepletion, 15 μL of a 1:1 slurry of protein G–agarose (Life Technologies) was incubated with the nuclear extracts for 60 min. The immune complexes were removed by centrifugation, and the nuclear extract was assayed for AP-1-binding activity by electrophoretic mobility shift assays [18]. Gel shift analysis for NF-κB was performed with 5 μg of nuclear extracts.

2.6. Isolation of microsomal proteins

Hepatic microsomal fractions prepared by differential centrifugation were washed in pyrophosphate buffer and stored in 50 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The subcellular preparations were stored at −70° until use.

2.7. Immunoblot analysis

SDS–PAGE and immunoblot analysis were performed according to previously published procedures [10,21]. Microsomal proteins were separated by 8% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with anti-rat mEH antibody, followed by incubation with biotinylated secondary antibody, and developed using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium [21]. The antibody was raised in rabbits by injecting a purified mEH protein that was characterized by N-terminal sequencing and peptide mapping analyses. Mobility of the band recognized by the antibody was confirmed by using the purified mEH protein and the authentic anti-mEH antibody. The specificity of the mEH antibody has been confirmed by a series of previous studies [11,12,22].

2.8. Preparation of a cDNA probe for mEH

A specific cDNA probe for the *mEH* gene was amplified by the reverse transcription–polymerase chain reaction using the selective primers [10,22] and was cloned in the pGEM+T vector (Promega).

2.9. Northern blot hybridization

Total RNA was isolated using the improved single-step method of thiocyanate–phenol–chloroform RNA extraction, as described by Puissant and Houdebine [23]. Northern blot analysis was carried out according to the procedures described previously [10]. Briefly, total RNA isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80° for 2 hr. The blot was incubated with hybridization buffer containing 50% deionized formamide, 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidine, and 0.1% BSA [Pentex Fraction V]), 0.1% SDS, 200 μg/mL of sonicated salmon sperm DNA, and 5× SSPE (1× SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.4) at 42° for 1 hr without probe. Hybridization was performed at 42° for 18 hr with a heat-denatured cDNA probe, which was random prime-labeled with [α-^{32}P]dCTP. Filters were washed twice in 2× standard saline citrate and 0.1% SDS for 10 min at room temperature and twice in 0.1× standard saline citrate and 0.1% SDS for 10 min at room temperature. Filters were finally washed in the solution containing 0.1× standard saline citrate and 0.1% SDS for 60 min at 60°. After quantitation of mRNA levels, the membranes were stripped and rehybridized with a ^{32}P-labeled cDNA probe complementary to 18S rRNA to quantify the amount of RNA loaded onto the membranes.

2.10. Data analysis

Scanning densitometry was performed with a Microcomputer Imaging Device, Model M1 (Imaging Research). One-way analysis of variance (ANOVA) procedures were used to assess significant differences between treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Morphological and molecular alterations during PCM

Light microscopic examinations of control livers showed sinusoids separated from hepatocytes, portal triads, and central veins (Fig. 1A). PCM elicited marked decreases in cytoplasmic eosinophilic contents with shrinkage of nuclei in the hepatocytes (Fig. 1B). Hepatic GSH content was determined in rats fed the low protein diet. The hepatic reduced glutathione level was decreased to 1.62 μmol/g liver wet weight after 4 weeks of protein restriction from 4.17 μmol/g liver wet weight in control rats (Table 1). Thus, PCM caused a 61% reduction in the hepatic GSH level relative to control.

Activation of AP-1 and/or NF-κB is associated with cellular oxidative stress and an altered redox state. The transcriptional factors regulate expression of the associated genes [24,25]. Studies were extended to determine whether the transcriptional factors were activated during PCM. Gel shift retardation analysis revealed that a nuclear AP-1 tran-

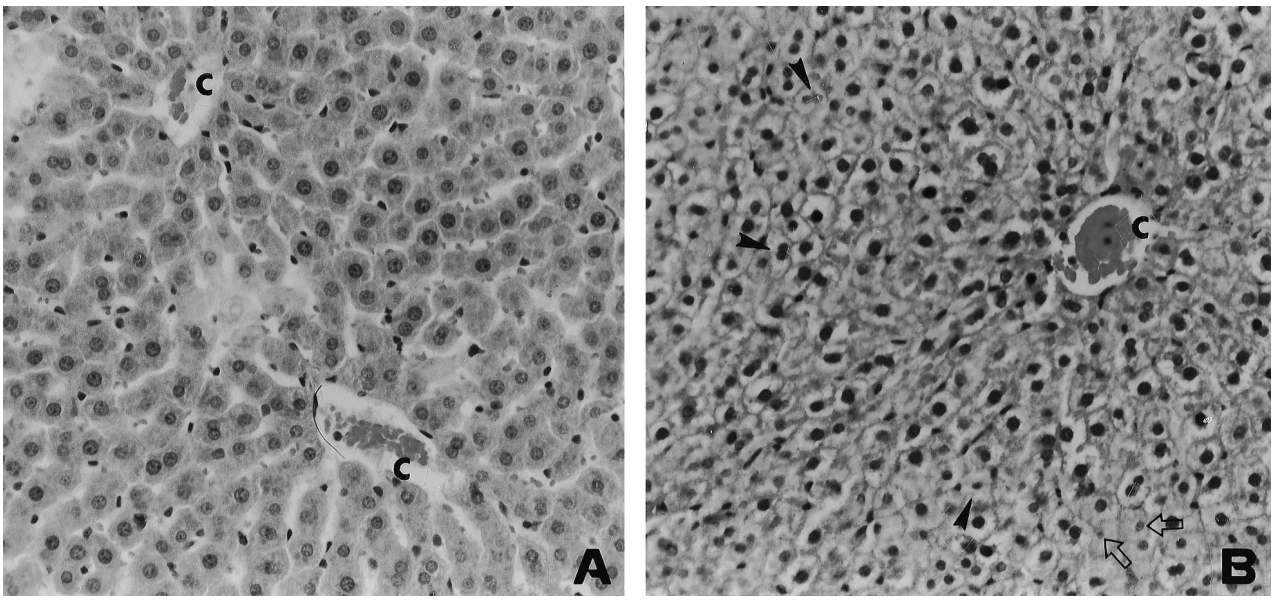

Fig. 1. Hepatic morphology of PCM rats. Hematoxylin and eosin-stained sections were photographed. (A) Normal liver from a control rat (×200). (B) Liver from a PCM rat (×200). Decreases in cytoplasmic eosinophilic contents (➤) and nuclear shrinkage are noted (➪). c, central vein. Results were confirmed by multiple analyses.

scription complex was activated in the rat liver during PCM (Fig. 2A). The specificity of the DNA probe to the PCM-activated AP-1-binding complex was supported by competition for binding to a radiolabeled AP-1 probe with a 10- to 20-fold molar excess of unlabeled AP-1 oligonucleotide, but not with an excess of unlabeled specific protein-1 or AP-2 oligonucleotide (Fig. 2A). Immunodepletion of AP-1 activity by the specific antibodies has been utilized to characterize the components of AP-1 proteins [18–20]. To identify the factor(s) that make(s) up inducible AP-1 activity, highly specific antibodies directed against individual AP-1 proteins were evaluated for their ability to inhibit DNA-binding activity. Reaction mixtures were incubated with 10 μg of the specific anti-c-Jun, anti-JunB, anti-JunD, anti-c-Fos, anti-FosB, or anti-Fra-1 antibodies prior to gel shift analysis (Fig. 2B). The immunodepletion study showed that the band intensity of the migrating complex with the AP-1 consensus sequence was decreased by the presence of specific antibodies against c-Jun protein (Fig. 2B). Either anti-c-Fos or anti-Fos-B antibody weakly, if at all, affected the AP-1 DNA binding. These results showed that the activation of AP-1 during PCM primarily involves c-Jun protein. In contrast to the activation of AP-1, NF-κB was not activated during PCM (Fig. 2C).

mEH expression was assessed in the rat liver by Western blot analysis. PCM caused a 3.7-fold induction of mEH in the liver (Fig. 3A, Table 2). Northern blot analysis was carried out to establish whether the induction of mEH was accompanied by a parallel increase in the mRNA (Fig. 3B). PCM elicited a 20-fold increase in the mEH mRNA level as compared to control.

Table 1
The hepatic glutathione content during PCM

Treatment	GSH (μmol/g liver wet weight)	% Control
Control	4.17 ± 0.49	100
PCM	1.62 ± 0.67*	39
Control + cysteine	5.93 ± 1.18	142
PCM + cysteine	7.15 ± 1.27†	171

Animals were fed the diet containing 23% or 5% casein for 4 weeks. Cysteine was gavaged at the dose of 250 mg/kg twice per day for the last 7 days during the 4-week period. The values are means ± SD (N = 6 animals). One-way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test.

* $P < 0.01$, significance as compared to control.

† $P < 0.01$, significance as compared to PCM.

3.2. Effects of cysteine supplementation

In contrast to the altered morphology of hepatocytes in PCM rats, a week of cysteine supplementation to PCM rats resulted in only mild fat degeneration with well-preserved portal structures and Kupffer cells in the liver (Fig. 4A). Methionine also prevented changes in cellular structure during PCM, showing a moderately decreased cytoplasmic eosinophilic content and a relatively decreased size of nuclei (Fig. 4B). Both the portal structure and the shape of Kupffer cells appeared to be well preserved. Cysteine appeared to be more effective than methionine at the same dose in preserving liver morphology. The hepatic GSH level was recovered by a week of cysteine supplementation in PCM rats, resulting in 170% of control. The increase in GSH content by cysteine was greater in rats fed the protein-deficient diet than in those fed the control diet (7.15 vs 5.93 μmol/g liver wet weight) (Table 1).

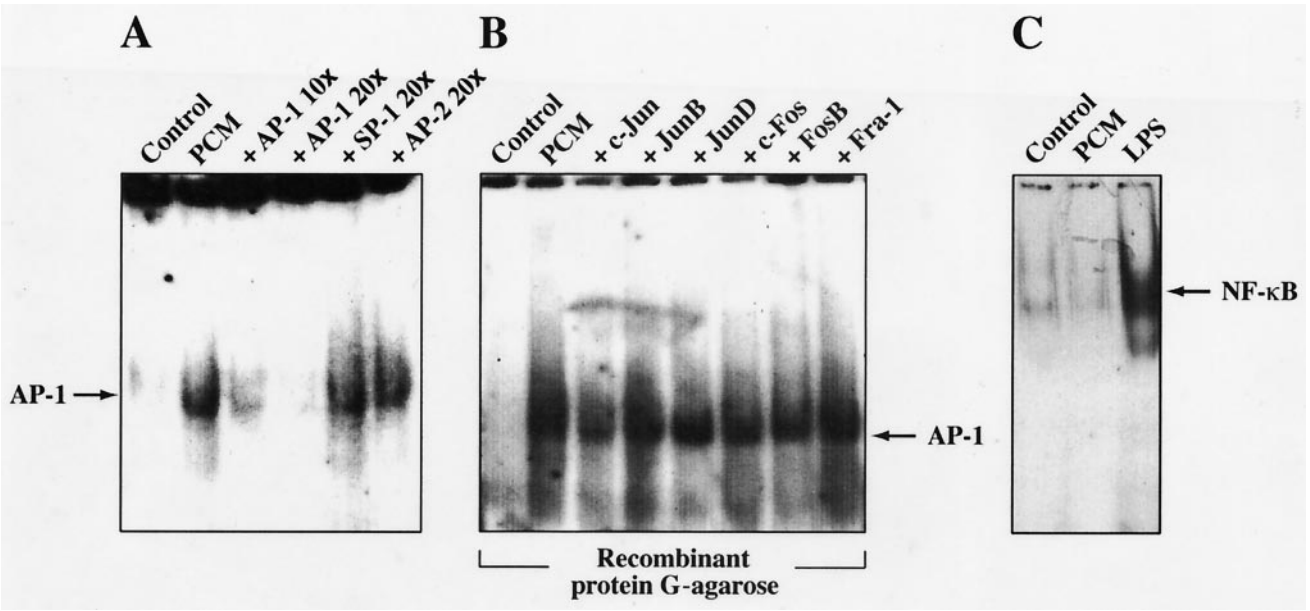

Fig. 2. Gel shift analysis of the AP-1 and NF-κB transcription complexes in hepatic nuclear extracts. (A) Hepatic nuclear extracts were isolated from rats fed a normal protein diet (control) or rats fed a low protein diet (PCM). All lanes contained 10 μg of nuclear extracts and 5 ng of radio-labeled AP-1 consensus sequence. Competition studies were carried out by adding a 10- or 20-fold excess of an unlabeled AP-1, specific protein-1, or AP-2 oligonucleotide before DNA-binding reactions. (B) Immunodepletion experiments were carried out by incubating the nuclear extracts from PCM rats with the polyclonal antibodies directed against c-Jun, JunB, JunD, c-Fos, FosB, or Fra-1 protein as described in Methods. (C) NF-κB transcription complexes. Rats intravenously injected with 0.1 μg/kg of lipopolysaccharide showed activation of nuclear NF-κB, whereas no signal was detected in control or PCM rats. Results were confirmed by repeated analyses.

The activation of the nuclear AP-1 complex was inhibited by cysteine supplementation in PCM rats (Fig. 5), indicating that the AP-1 activation resulted from cysteine deficiency. Addition of 10 mM cysteine to the nuclear extract with AP-1-binding activity, however, resulted in no change (data not shown). Thus, cysteine may not directly interact with the activated AP-1.

To determine whether the induction of mEH during PCM was related to cellular oxidative stress resulting from cysteine deficiency, mEH expression was assessed in PCM rats supplemented with cysteine. Interestingly, mEH induction during PCM was completely reversed by cysteine (Table 2). Methionine was also active at the same dose as cysteine. Thus, both cysteine and methionine were active in restoring the mEH level toward that of control (Fig. 6A). Treatment of rats fed the 23% casein diet with cysteine caused no alteration in enzyme expression. Northern blot analysis was carried out to establish whether the reversal of mEH induction was accompanied by a parallel change in the mRNA. The hepatic mEH mRNA level that increased during PCM completely returned to that of control following cysteine or methionine treatment (Fig. 6B).

Discussion

Studies in our laboratories have shown that plasma concentrations of therapeutic agents were elevated in PCM animals [6,7] and that the expression of major forms of P450 was suppressed by protein restriction [8]. Cytochrome P450 expression was partially or completely recovered by cysteine supplementation. The pharmacokinetic parameters were also restored by cysteine [8]. Other studies in this and other laboratories have demonstrated that rats fed the low protein diet are more susceptible to the toxicities of xenobiotics such as 4-methylthiazole, pyridine* and rifampicin [4,26]. Histopathological examinations in the present study revealed that PCM rats showed marked decreases in eosinophilic contents in the cytoplasm and shrinkage of the nuclei in hepatocytes. These morphological changes, however, were completely prevented by cysteine, and partially by methionine supplementation. Hence, cysteine appeared to be the amino acid primarily responsible for preserving the morphological structures as well as the hepatocellular function. Toxicities induced by xenobiotics such as 4-methylthiazole were also minimized by cysteine pretreatment in PCM animals.†

Serum concentrations of citrulline, cysteine, ornithine, taurine, and tyrosine were lower in rats fed the 5% casein diet compared with animals consuming the normal diet [2]. Because cysteine is a direct precursor of GSH, the liver GSH content would be related to the intake of the sulfur amino acid. In the present study, the hepatic GSH content decreased after rats were fed a low protein diet, which is in

* Cho MK and Kim SG, unpublished data.
† Cho MK and Kim SG, unpublished data.

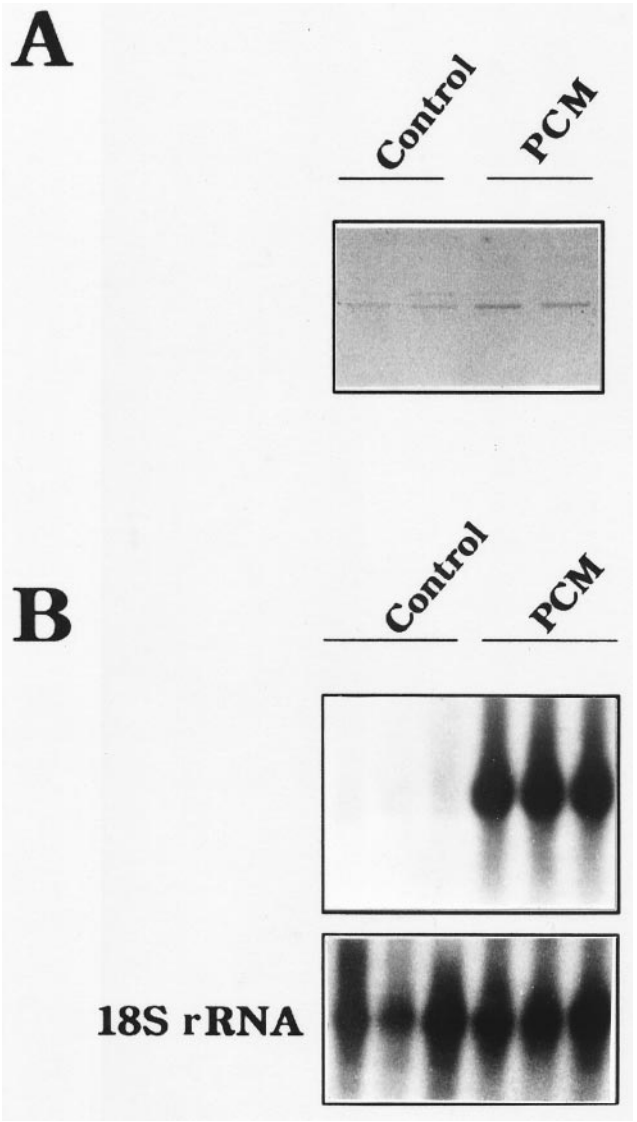

Fig. 3. Expression of mEH during PCM. (A) Immunoblot analyses of hepatic mEH protein. The representative immunoblots show mEH protein levels in the liver microsomes, isolated from rats fed the 23% (control) or 5% (PCM) casein diet for 4 weeks. Each lane was loaded with 5 μg of proteins. (B) Northern blot analysis of mEH mRNA in PCM rats. The mEH mRNA level was determined in total RNA fractions (20 μg each) isolated from rats fed the 23% (control) or 5% (PCM) casein diet for 4 weeks. The amount of RNA loaded in each lane was assessed by rehybridization of the stripped membrane with a ^{32}P-labeled probe for 18S rRNA. Relative changes in the mEH mRNA level were assessed by scanning densitometry of Northern blots.

agreement with other reports [27,28]. This shows that GSH turnover may be directly affected by protein intake. Depletion of hepatic GSH increases the susceptibility of animals to free radical-induced tissue damage, because liver GSH plays a critical role in the detoxification of oxidative metabolites produced from endogenous and exogenous substances. The present study showed that the hepatic GSH content was increased by cysteine supplementation in rats fed either the normal protein diet or the protein-deficient diet. Cysteine would be converted to intracellular GSH in the liver, which plays a critical role in the detoxification of reactive intermediates of oxidative metabolism [28]. A greater increase in hepatic GSH content was observed in PCM rats. The membrane transporting system seemed to be important in maintaining the appropriate cellular redox state, presumably via reduced GSH. Cysteine is transported into the cells primarily by the Na^+-dependent carrier system in the liver [29]. Cysteine availability to hepatocytes has been shown to be greater from exogenous cysteine than from methionine [28].

Both AP-1 and NF-κB have been implicated as transcriptional factors activated by cellular oxidative stress [24]. In the present study, the nuclear AP-1 transcription complex was activated in the liver of PCM rats, although NF-κB failed to be activated. Results of the present study support the notion that PCM elicits oxidative stress in hepatocytes, as evidenced by the reduction in glutathione level and AP-1 activation, which may result in part from deficiency of cysteine and/or cysteine metabolite(s). The similarity between the antioxidant response element and the AP-1-binding motif has been raised, although the consensus sequence for AP-1 binding is distinct from that of the antioxidant response element [30]. Studies were extended to determine the factors that make up inducible AP-1 activity. The observation that the AP-1-binding activity was reduced by incubation of the nuclear extracts with the antibody directed against c-Jun protein provided evidence that the latter is involved in the activation of nuclear AP-1 during PCM. The expression of γ-glutamylcysteine synthetase is increased by GSH depletion with the activation of the Jun/AP-1 transcription factor [31,32]. Recent studies have shown that the activated oxygens seem to be potentially responsible for the transcriptional induction of detoxifying enzymes in associ-

Table 2
Relative hepatic mEH protein level in PCM rats with or without cysteine/methionine supplementation

	Untreated		Cysteine		Methionine	
	−PCM	+PCM	−PCM	+PCM	−PCM	+PCM
mEH	1	3.7 ± 0.6*	1.0 ± 0.3	1.3 ± 0.4†	1.0 ± 0.4	1.0 ± 0.3†

The relative changes in hepatic mEH level were quantified in rats fed the 23% (−PCM) or 5% (+PCM) casein diet for 4 weeks. Cysteine or methionine (250 mg/kg body weight twice per day) was supplemented for the last 7 days during the 4-week period. Band intensities were assessed by scanning densitometry of the immunoblots. Data represent the means ± SD from 6 separate experiments. One-way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test (control level in rats fed the normal diet = 1).

* $P < 0.01$, significance as compared to rats fed the normal diet.

† $P < 0.01$, significance as compared to PCM rats.

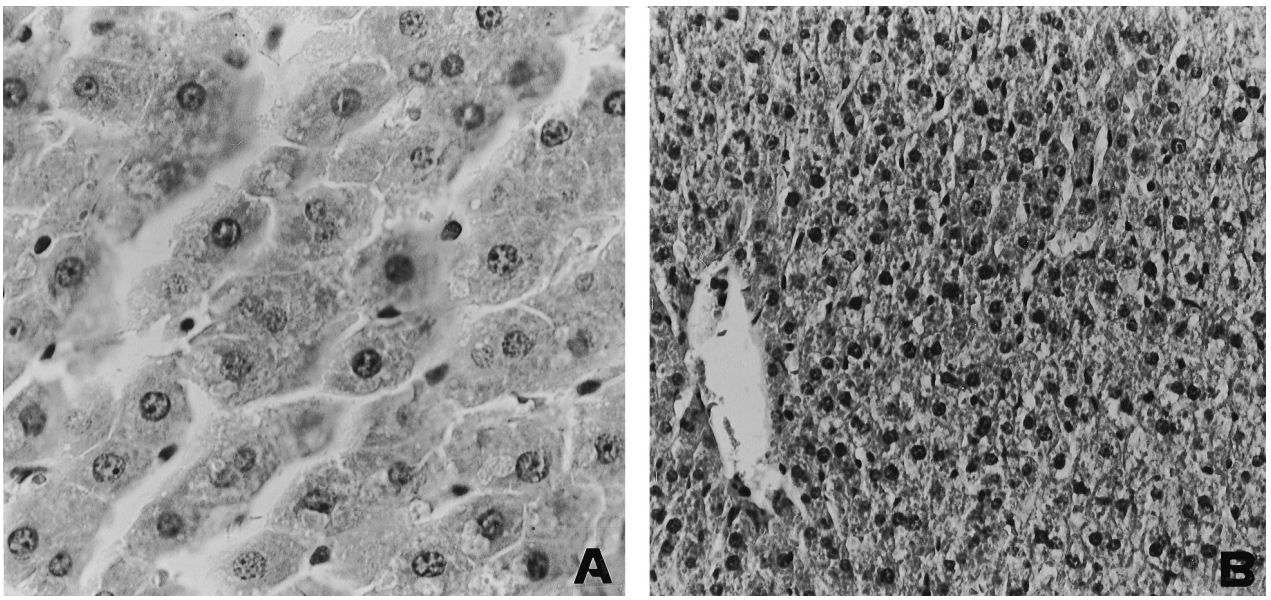

Fig. 4. Hepatic morphology of PCM rats with or without cysteine or methionine supplementation. Hematoxylin and eosin-stained sections were photographed. (A) Liver from a PCM rat supplemented with cysteine (×400). Greater magnification was used to clearly show the improvement in hepatocyte morphology. (B) Liver from a PCM rat with methionine supplementation (×200).

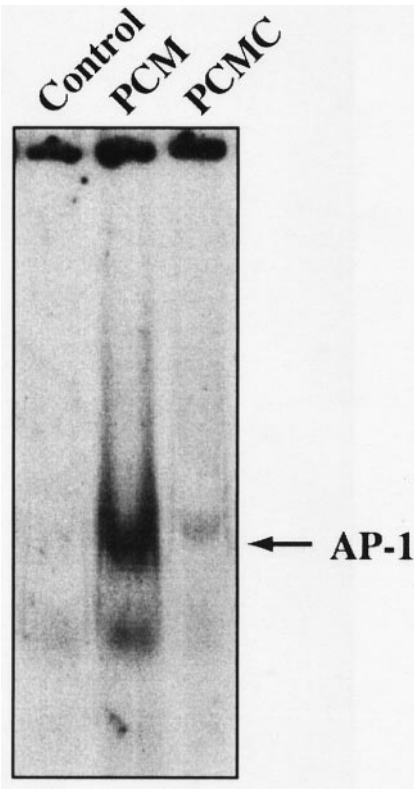

Fig. 5. AP-1-binding activity after cysteine supplementation. Gel shift analysis was carried out with hepatic nuclear extracts from control, PCM rats, or PCM rats supplemented with cysteine (PCMC) (250 mg/kg body weight twice per day) for 7 days. Each reaction contained 10 μg of nuclear extract and 5 ng of radio-labeled AP-1 consensus sequence. Results were confirmed by repeated analyses.

ation with Nrf and Jun proteins [33]. Phosphorylation of the amino-terminal transactivating domain by the c-Jun N-terminal kinases of mitogen-activating protein kinases affects AP-1 activation [34–36]. Studies in this laboratory revealed that cellular kinase signal transduction pathways in H4IIE cells stimulated by oxidative stress involve p38 kinases.* Involvement of cysteine in the activation of AP-1 was supported by the reversal of AP-1 activation in response to cysteine supplementation. However, activation of AP-1 was not abolished *in vitro* in the presence of excess cysteine. Hence, cysteine would be involved upstream of AP-1 activation.

The cellular redox state affects the transactivation of oxygen-responsive genes [24,37,38]. Expression of the enzyme associated with the redox state would be affected by the GSH content in the liver after protein restriction. It is also possible that cysteine directly affects the sulfhydryl residues of critical proteins associated with gene regulation [15,39]. Studies using human hepatic microsomes have shown that variability exists in the expression of mEH [40,41]. The *mEH* gene appeared to be transcriptionally activated by reactive oxygen species produced from xenobiotic metabolism [11]. A study in our laboratory also showed that mEH is inducible by γ-ray ionizing radiation with a marked increase in the mRNA. Transcriptional activation of the hepatic *mEH* gene in response to antioxidants may be mediated with AP-1 and/or the antioxidant response element. The transcriptional activation of the *mEH* gene may result from the change in redox state in association

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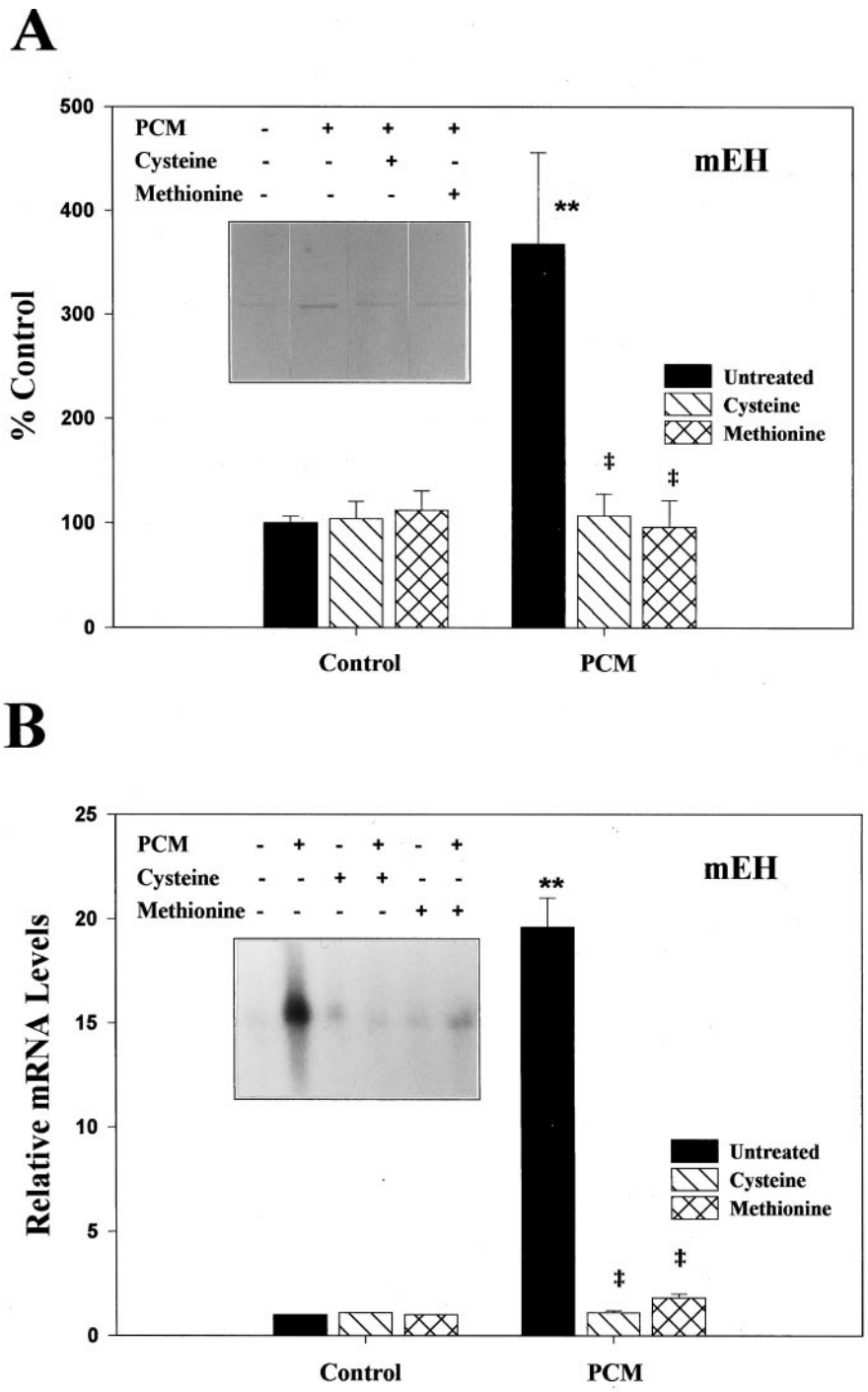

Fig. 6. mEH protein and mRNA levels in PCM rats with or without cysteine/methionine supplementation. (A) Immunoblot analyses of hepatic mEH protein. The representative immunoblots show mEH protein levels in liver microsomes isolated from rats fed the 23% (control) or 5% (PCM) casein diet for 4 weeks with or without cysteine/methionine supplementation. Cysteine or methionine was supplemented for the last 7 days during 4 weeks of protein restriction. Each lane was loaded with 5 μg of proteins. (B) Northern blot analyses for the mEH mRNA were carried out with total RNA fractions (20 μg each) isolated from rats fed the 23% (control) or 5% (PCM) casein diet for 4 weeks with or without cysteine/methionine supplementation. Changes in the mEH mRNA level relative to control were assessed by scanning densitometry. Data represent the means ± SD from 6 separate experiments. One-way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test ($^{**}P < 0.01$, significant as compared to rats fed the normal diet; $^{‡}P < 0.01$, significant as compared to rats fed the protein-deficient diet) (control mRNA level = 1).

with the intracellular GSH level. AP-1 activation during protein deprivation is likely to affect the expression of the detoxifying enzyme in the liver. The current study revealed that mEH was induced with a 20-fold increase in the mRNA level in the liver of PCM rats. Both cysteine and methionine were effective in preventing the induction of mEH and the

elevation of the mRNA during PCM. The current results showed that induction of the phase II enzyme by protein deprivation may be associated with the activation of nuclear c-Jun/AP-1, and that both c-Jun/AP-1 activation and mEH expression were modulated by cysteine. That there was no alteration of hepatic mEH expression in rats fed the normal protein diet by cysteine was in agreement with the previous observation that an excess protein diet did not produce a higher GSH content in the liver [27]. Cysteine at the daily dose of 50 mg/kg was also active in preventing enzyme induction. The reasonable estimated biologic requirements of methionine and cystine are 58 and 27 mg/kg body weight per day for the infant and preschool child, respectively [1]. Considering the adequacy of the sulfur amino acid requirements for the infant and child and the dose employed in the present study, the basis for predicting the minimum physiologic intake necessary to maintain body amino acid balance for correcting the changes during PCM may be extended to human subjects.

The results of this study provide strong evidence that PCM elicited morphological changes in the liver with a marked decrease in reduced glutathione and activated c-Jun/AP-1, which might result from oxidative stress in hepatocytes. The mEH enzyme was induced with a substantial increase in the mRNA during PCM. Among the essential amino acids decreased, cysteine may play an essential role in preserving the morphology and physiologic function of hepatocytes as well as in regulating adaptive gene expression during PCM.

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