Differential regulation of γ-glutamylcysteine synthetase heavy and light subunit gene expression

Jiaxin CAI, Zong-Zhi HUANG and Shelly C. LU[1]

Division of Gastrointestinal and Liver Diseases, Department of Medicine, University of Southern California School of Medicine, Los Angeles, CA 90033, U.S.A.

γ-Glutamylcysteine synthetase (GCS) is the rate-limiting enzyme in the biosynthesis of glutathione and is composed of a heavy and a light subunit. Although the heavy subunit is enzymically active alone, the light subunit plays an important regulatory role by making the holoenzyme function more efficiently. In the current study we examined whether conditions which are known to influence gene expression of the heavy subunit also influence that of the light subunit, and the mechanisms involved. Treatment of cultured rat hepatocytes with hormones such as insulin and hydrocortisone, or plating hepatocytes under low cell density increased the steady-state mRNA level of the heavy subunit only. Treatment with diethyl maleate (DEM), buthionine sulphoximine (BSO) and t-butylhydroquinone (TBH) increased the steady state mRNA level and gene transcription rates of both subunits. These treatments share in common their ability to induce oxidative stress and activate nuclear factor κB (NF-κB). Treatment with protease inhibitors 7-amino-1-chloro-3-tosylamido-2-heptanone (TLCK) or L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) had no influence on the basal NF-κB and GCS subunit mRNA levels, but blocked the activation of NF-κB by DEM, BSO and TBH, and the increase in GCS heavy subunit mRNA level by BSO and TBH. On the other hand, the DEM-, BSO- and TBH-induced increase in GCS light-subunit mRNA level was unaffected by TLCK and TPCK. Thus only the heavy subunit is hormonally regulated and growth sensitive, whereas both subunits are regulated by oxidative stress. Signalling through NF-κB is involved only in the oxidative-stress-mediated changes in the heavy subunit gene expression.

INTRODUCTION

Glutathione (GSH) is a tripeptide, γ-glutamylcysteinylglycine, which is synthesized by virtually all mammalian cells. GSH plays a vital role in defence against toxins and free radicals, and in storing and transferring cysteine [1]. The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is rate-limiting and catalysed by γ-glutamylcysteine synthetase (GCS) which is regulated physiologically by feedback competitive inhibition by GSH and by the availability of cysteine [1,2]. GCS is composed of a heavy ($M_r \sim 73000$) (GCS-HS) and a light ($M_r \sim 30000$) subunit (GCS-LS), which are encoded for by different genes and dissociate under reducing conditions [3,4]. The heavy subunit exhibits all of the catalytic activity of the isolated enzyme as well as feedback inhibition by GSH [5]. The light subunit is enzymically inactive but plays an important regulatory function by lowering the K_m of GCS for glutamate and raising the K_i for GSH [4,6]. In many conditions (i.e. drug-resistant tumour cell lines, oxidative stress, treatment with anti-oxidants or GSH-conjugating agents) where GCS activity is increased, there is also an increase in the GCS-HS mRNA level [7–12]. We have shown also, in primary cultures of rat hepatocytes, that the activity of GCS can be induced by insulin (1 μg/ml) or hydrocortisone (50 nM) treatment [13], and by lowering the cell density of the initial plating [14]. Recently, we demonstrated that the mechanism of these effects was an increase in the transcription of GCS-HS [15]. Thus regulation of the GCS-HS gene expression appears critical for GSH homoeostasis.

There are controversial reports regarding the effect of buthionine sulphoximine (BSO) on GCS-HS mRNA level [12,16,17]. One report showed no change in the mouse hepatic GCS-HS mRNA level when GSH was depleted to 30% of controls [12], whereas two other reports showed increased GCS-HS mRNA levels in peripheral mononuclear cells of patients [16] and cultured human T lymphocytes [17] after treatment with BSO. While much is known about the regulation of the heavy subunit, virtually nothing is known about the regulation of the light subunit. Since the GCS-LS plays a critical regulatory role on the overall function of the enzyme, it is important to understand its gene regulation. In this report we examined whether factors that influence the heavy subunit gene expression also influence that of the light subunit, clarified the effect of BSO on the gene expression of both GCS subunits and identified the possible signalling mechanism involved.

MATERIALS AND METHODS

Materials

GSH, collagenase (type IV), NADPH, 5,5′-dithiobis(2-nitrobenzoic acid), diethyl maleate (DEM), BSO, GSH reductase, insulin, hydrocortisone, fetal bovine serum, t-butylhydroquinone (TBH), 7-amino-1-chloro-3-tosylamido-2-heptanone (TLCK) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's minimal essential/Ham's F12 medium was

Abbreviations used: ANOVA, analysis of variance; GCS, γ-glutamylcysteine synthetase; GSH, reduced glutathione; GCS-HS, GCS heavy subunit; GCS-LS, GCS light subunit; DEM, diethyl maleate; BSO, D,L-buthionine sulphoximine; TBH, t-butylhydroquinone; TLCK, 7-amino-1-chloro-3-tosylamido-2-heptanone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; NF-κB, nuclear factor-κB.

[1] To whom correspondence should be addressed.

purchased from Mediatech's Cellgro (Tustin, CA, U.S.A.). [^{32}P]dCTP (3000 Ci/mmol), [^{32}P]UTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from New England Nuclear (DuPont, Boston, MA, U.S.A.). The total RNA isolation kit was obtained from Promega (Madison, WI, U.S.A.). All other reagents were of analytical grade and were obtained from commercial sources.

Animals

Male Sprague–Dawley rats (Harlan Laboratory Animals, Inc., San Diego, CA, U.S.A.), weighing 260–350 g were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum*. All animals received humane care in compliance with the National Research Council's criteria for humane care as outlined in 'Guide for the Care and Use of Laboratory Animals', prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication no 86-23, revised 1985).

Preparation and treatment of cultured hepatocytes

Hepatocytes were isolated from fed male Sprague–Dawley rats (260–350 g) as described previously [15,18]. Initial cell viability was $\geqslant 90\,\%$, determined by 0.2 % Trypan Blue exclusion. The plating medium was Dulbecco's minimal essential/Ham's F12 with high glucose content (3151 mg/l), supplemented with methionine (1 mM), 10 % (v/v) fetal bovine serum, insulin (1 μg/ml) and hydrocortisone (50 nM). Both insulin and hydrocortisone were omitted when the effects of hormones and density were examined. High-density plating of cells (0.6×10^5 cells/cm^2) was used to examine the effect of hormones, DEM, BSO and TBH. Low-density plating was 0.1×10^5 cells/cm^2. Cells were plated on 60×15-mm dishes precoated with rat tail collagen using 5 ml of plating medium and the dishes were incubated at 37 °C in 5 % CO_2/95 % air. Two hours after plating, the medium was changed to remove dead, unattached cells and was replaced with medium without fetal bovine serum. Cell attachment averaged $\sim 60\,\%$.

All treatment of cultured cells were carried out within 24 h of plating. To examine the effect of hormones and cell density, cells were plated at low or high density and high-density cells were treated with either insulin (1 μg/ml), hydrocortisone (50 nM) or vehicle for 24 h. To examine the effect of DEM, the day after plating cells were treated with 0.2 mM DEM or vehicle for 20 min, followed a change to Dulbecco's minimal essential/Ham's F12 medium for 3–6 h. To examine the effect of BSO, cells were treated with 0.1–5 mM BSO or vehicle for 12 h and in some cases this was followed by a change to Dulbecco's minimal essential/Ham's F12 medium for an additional 6 h. To examine the effect of TBH, cells were treated with 50 μM TBH or vehicle for 6–24 h. At the end of the treatment, cells were processed for GSH measurement by the recycling method of Tietze [19], RNA extraction, and in some cases, for the estimation of lactate dehydrogenase activity of the medium as described previously [20].

To examine the effect of the protease inhibitors TLCK and TPCK on the basal GCS subunit mRNA levels, cells were treated with TLCK (300 μM), TPCK (20 μM) or vehicle control for 12 h. Higher doses of TPCK ($\geqslant 30\,\mu$M) induced cell lysis. To examine the effect of TLCK and TPCK on TBH-, BSO- and DEM-induced changes in GCS subunit mRNA levels, cultured rat hepatocytes were pretreated with TLCK (300 μM), TPCK (20 μM) or vehicle for 30 min before the addition of TBH (50 μM for 12 h) or BSO (1 mM for 12 h), or were pretreated for 12 h before the addition of DEM (0.2 mM for 20 min). TLCK and TPCK were left in the medium while cells were treated with TBH, BSO or DEM. At the end of BSO or DEM treatment the medium was changed and the cells were processed for cell GSH measurement, RNA or nuclear protein extraction, 6 h later.

Nucleic acid extraction

Total RNA was isolated from cultured rat hepatocytes after the treatments described above according to the method of Chomczynski and Sacchi [21]. Poly(A)-RNA (mRNA) was isolated using oligo(dT) cellulose columns according to protocol provided by Life Technologies (Grand Island, NY, U.S.A.). The RNA concentration was determined spectrophotometrically before use and in the case of total RNA, the integrity was checked by electrophoresis with subsequent ethidium bromide staining.

Northern hybridization analysis

Northern hybridization analysis was performed on total RNA (20–30 μg) and poly(A)-RNA (3 μg) using standard procedures [22] as described previously [15]. The GCS-HS cDNA probe is composed of a 390 bp fragment corresponding to nucleotides 79–468 of the rat kidney GCS-HS sequence reported previously [3], and the GCS-LS cDNA probe is composed of a 1.1 kb fragment corresponding to nucleotides 122–1232 of the rat kidney GCS-LS sequence published by [4]. Both were labelled with [^{32}P]dCTP using a random-primer kit (Primer-It II Kit; Stratagene, La Jolla, CA, U.S.A.). To ensure equal loading of RNA samples, the same membrane was rehybridized with a ^{32}P-labelled human β-actin cDNA probe (Clontech, Palo Alto, CA, U.S.A.). Autoradiography and densitometry (Gel Documentation System, Scientific Technologies, Carlsbad, CA, U.S.A. and an NIH Image 1.60 software program) were used to quantify relative RNA. In the case of GCS-LS, where there are multiple mRNA species, all the bands were quantified and the total was used for comparison. The results of Northern-blot analyses were normalized to β-actin.

Nuclear run-on transcription assay

Nuclei isolation and nuclear run-on assay were carried out as described previously [15]. Briefly, nuclei were isolated from cultured rat hepatocytes after various treatments, 2×10^7 nuclei (determined using a haemocytometer) were resuspended in 50 μl nuclei-freezing buffer [50 mM Tris/HCl, pH 8.0/50 % (v/v) glycerol/5 mM $MgCl_2$/0.1 mM EDTA], snap-frozen in liquid N_2 and stored at -80 °C. After nuclear run-on assay using the isolated nuclei, identical amounts of labelled nuclear RNAs [$(4–5) \times 10^7$ cpm] were hybridized overnight with plasmid DNAs (5 μg each) consisting of GCS-HS cDNA in pBluescript® II SK (+/−) or human β-actin cDNA in pBluescript® II SK (+/−) or GCS-LS cDNA (200 ng) alone with a slot–blot apparatus (Bio-Dot® SF, Bio-Rad, Hercules, CA, U.S.A.) as described previously [15]. After hybridization, filters were washed and subjected to autoradiography and densitometry as described above.

Electrophoretic mobility-shift and supershift assays for nuclear factor-κB

Electrophoretic mobility-shift and supershift assays for nuclear factor-κB (NF-κB) were carried as described previously [23,24]. Nuclear protein extracts were obtained after various treatments as described [25]. Nuclear protein (10–15 μg) was preincubated with 2 μg of poly(dI-dC) in a buffer containing 10 mM Hepes, pH 7.6/50 mM KCl/0.1 mM EDTA/1 mM dithiothreitol/5 mM $MgCl_2$/10 % (v/v) glycerol for 10 min on ice. ^{32}P-end-

labelled double-stranded oligonucleotide (0.2–0.5 ng; 30000–50000 c.p.m.) containing the binding site for NF-κB (top strand: 5′-GCAGAGGGGACTTTCCGAGA-3′; bottom strand: 5′-GTCTCGGAAAGTCCCCTCTG-3′) was then added with or without 100-fold excess of unlabelled probe. Mixtures were incubated for 30 min on ice, loaded on to a 6 % non-denaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris/45 mM borate/0.5 mM EDTA, pH 8.0. For antibody supershift assays, 1.5 μl of anti-p65 or anti-p50 antibodies (Santa Cruz Biotech, Inc., Santa Cruz, CA, U.S.A.) was added to respective samples after 30 min of incubation with the labelled probe, and all samples were further incubated for another 45 min on ice before electrophoresis. Gels were dried and subjected to autoradiography.

Statistical analysis

For cultured cells, each cell preparation was derived from one animal and multiple plates were used for each treatment and each time point for RNA extraction. Duplicate plates were used for GSH measurement. The mean of each duplicate from one experiment was considered as $n = 1$ and the means of multiple experiments for treatment and control groups were compared by one-way analysis of variance (ANOVA) followed by Fisher's test. For cell GSH levels, actual values were compared. For changes in mRNA levels, ratios of GCS-HS or GCS-LS to β-actin densitometric values were compared. The criterion for significance was $P < 0.05$. Results are shown as means $\pm$ S.E. of the number of experiments (n).

RESULTS

Effect of hormones and cell density on GCS subunit mRNA level

We first examined the effect of hormones and plating density, which we had previously shown to increase the steady-state mRNA level of GCS-HS [15], on that of GCS-LS. Figure 1 shows that treatment of cultured rat hepatocytes with insulin (1 μg/ml) or hydrocortisone (50 nM), or low-density plating increased the steady-state mRNA level of the GCS-HS without affecting that of the GCS-LS.

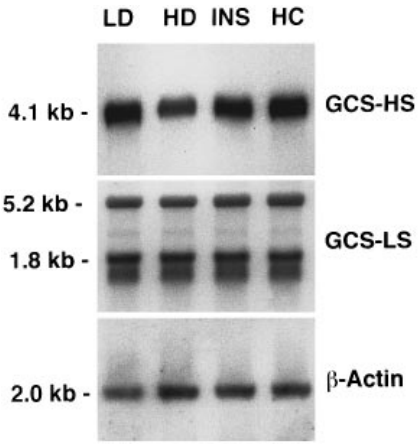

Figure 1 Effect of insulin, hydrocortisone and initial plating cell density on the steady-state mRNA level of GCS subunits

Poly(A)-RNA (3 μg each lane) samples obtained from cultured rat hepatocytes plated at low density (LD) or high density (HD) and treated with insulin (INS, 1 μg/ml), hydrocortisone (HC, 50 nM) or vehicle (HD) were analysed by Northern-blot hybridization with GCS-HS, GCS-LS and β-actin cDNA probes as described in the Materials and methods section. The same membrane was sequentially hybridized with all three probes. A representative Northern blot from 4 separate experiments is shown.

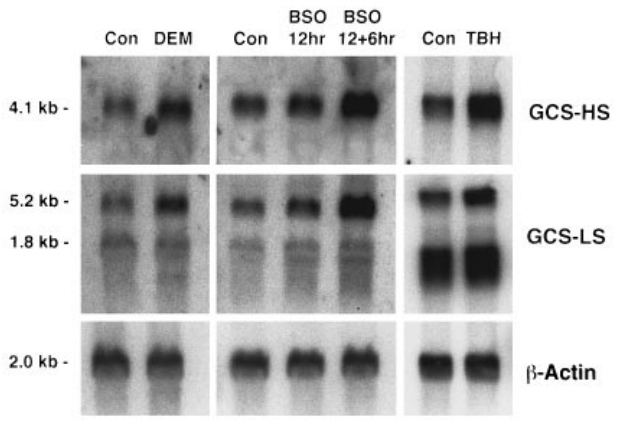

Figure 2 Effect of DEM, BSO and TBH on the steady-state mRNA level of GCS subunits

Poly(A)-RNA (3 μg each lane) samples obtained from cultured rat hepatocytes treated with DEM (0.2 mM for 20 min followed by a change of medium for 6 h), BSO (5 mM for 12 h $\pm$ medium change for an additional 6 h) or TBH (50 μM for 12 h) were analysed by Northern-blot hybridization with GCS-HS, GCS-LS and β-actin cDNA probes as described in the Materials and methods section. Con, control sample. The same membrane was sequentially hybridized with all three probes. A representative Northern blot from 3–6 separate experiments is shown.

Effect of DEM, BSO, and TBH on GCS subunit mRNA level

Treatment of cultured rat hepatocytes with DEM, BSO and TBH increased the steady-state mRNA level of both GCS subunits. Treatment with 0.2 mM DEM for 20 min depleted cell GSH to 30 % of controls. By 3 and 6 h after change of medium, the cell GSH level had recovered to 65 % and 100 %, respectively, of controls. GCS-HS mRNA level increased to 35 % at 3 h (results not shown) and to 70 % at 6 h after the medium change (Figure 2). GCS-LS mRNA level also increased after DEM treatment to similar levels as the heavy subunit (Figure 2).

Treatment with 5 mM BSO for 12 h depleted cell GSH to 10 % of controls and GSH continued to fall to 5 % of controls 6 h later despite a medium change at 12 h, to remove BSO (12 + 6 h). The steady-state mRNA level of both subunits of GCS was increased by 50–55 % after 12 h of BSO treatment (BSO 12 h) and by 110–150 % over control 6 h after the medium change (BSO 12 + 6 h) (Figure 2). When we examined the relationship of percentage control cell GSH and GCS subunit mRNA levels from cells treated with various concentrations of BSO (0.1–5 mM), we found that the steady-state mRNA level of either subunit did not increase significantly (i.e. > 150 % of control) until cell GSH was depleted to ~ 10 % of control level. Below 10 % of control cell GSH, the relationship appeared exponential (Figure 3).

Treatment with 50 μM TBH led to an initial lowering of cell GSH (69 % of control at 12 h), which was followed by recovery and then an increase over the control (132 % of controls at 24 h). The maximum effect on GCS subunit mRNA level was observed after 12 h of TBH treatment (Figure 2). None of the treatments caused an increase in cell lysis, measured by the release of lactate dehydrogenase into the culture medium, under these experimental conditions.

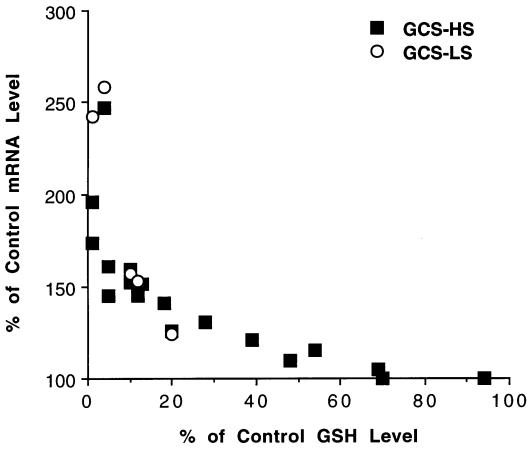

Figure 3 Relationship of control-cell GSH and GCS subunit mRNA levels

Cultured rat hepatocytes were treated with various concentrations of BSO (0.1–5 mM for 12 h and in some cases, an additional 6 h after medium change). GSH level represents the level at the end of treatment. The data from six experiments are shown for GCS-HS and three experiments for GCS-LS.

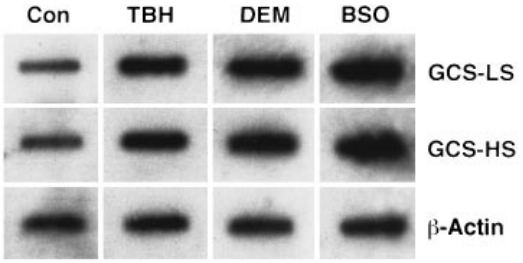

Figure 4 Effect of TBH, DEM and BSO on GCS subunit gene transcription in cultured rat hepatocytes

Cultured cells were treated with TBH (50 μM for 12 h), DEM (0.2 mM for 20 min followed by a change of medium for 6 h) or BSO (5 mM for 12 h followed by medium change for 6 h) and nuclei were isolated for nuclear run-on transcription assay as described in the Materials and methods section. Con, control sample. A representative slot–blot is shown from three separate experiments.

Mechanism of the increase in GCS subunit mRNA levels

We showed previously that the effect of hormones and cell density on the steady-state GCS-HS mRNA level was mediated at the level of gene transcription [15]. To see if the effect of DEM, BSO and TBH was also exerted at the level of gene transcription, a nuclear run-on assay was performed. Figure 4 shows that all three treatments increased the rate of gene transcription of both GCS subunits comparably. Table 1 summarizes the effects of these treatments on cell GSH, GCS subunit mRNA levels and the rate of gene transcription.

Involvement of NF-κB in the signalling mechanism

Recently, activation of NF-κB was shown to be the signalling mechanism for cytokine-induced stimulation of GCS-HS gene expression [26]. Since DEM, BSO and TBH are agents capable of inducing oxidative stress [12,27], which is known to activate NF-κB [26], we examined whether activation of NF-κB was involved in mediating the effect of these treatments on the gene expression of GCS subunits. Figure 5 shows a representative gel mobility-shift and supershift assay which confirms that all three treatments led to activation of NF-κB, which was blocked by the protease inhibitors TLCK and TPCK. Note that these protease inhibitors did not affect the basal level of NF-κB. We then examined the effect of TLCK and TPCK on TBH-, BSO- and DEM-induced increase in GCS subunit gene expression by measuring the steady-state mRNA level of the two subunits. Figure 6 is a representative Northern-blot analysis of these experiments. Both TLCK and TPCK blocked the TBH and BSO-induced increase in GCS-HS mRNA level significantly but the effect on DEM was not as clear (Figure 6A). On the other hand, TLCK and TPCK did not influence the effect of TBH, BSO or DEM on GCS-LS

Table 1 Effect of various treatments on cell GSH, GCS-subunit mRNA levels and gene transcription rates in cultured rat hepatocytes

Cultured rat hepatocytes were treated with insulin (1 μg/ml), hydrocortisone (50 nM), plated at low cell density, treated with DEM (0.2 mM for 20 min followed by change of medium for 6 h), BSO [5 mM for 12 h (BSO-12 h) or with medium change for an additional 6 h (BSO-12 + 6 h)], TBH (50 μM for 12 h) as described in the Materials and methods section. For GCS-HS mRNA level, Northern-blot analysis was done using total RNA for some experiments and poly(A)-RNA for others. For GCS-LS mRNA level, Northern-blot analysis was done using poly(A)-RNA. The same membranes were rehybridized with β-actin and the results were normalized to β-actin for comparison. Nuclear run-on assay was as described in the Materials and methods section. The results are the means ± S.E. of 3–6 experiments. *$P < 0.05$ versus control by ANOVA followed by Fisher's test.

		Percentage of control mRNA level		Gene transcription rate	
Treatment	Cell GSH	GCS-HS	GCS-LS	GCS-HS	GCS-LS
Insulin	171 ± 15*	223 ± 14*	107 ± 5		
Hydrocortisone	161 ± 12*	287 ± 63*	112 ± 9		
Low cell density	172 ± 8*	246 ± 35*	110 ± 14		
DEM-6 h	104 ± 7	171 ± 15*	157 ± 14*	208 ± 25*	212 ± 38*
BSO-12 h	10 ± 2*	150 ± 6*	155 ± 2*		
BSO-12 + 6 h	5 ± 1*	206 ± 20*	250 ± 8*	208 ± 27*	219 ± 26*
TBH-12 h	69 ± 4*	178 ± 22*	150 ± 17*	180 ± 17*	176 ± 15*

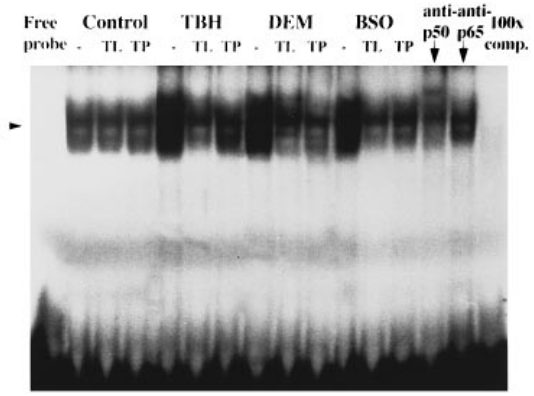

Figure 5 Electrophoretic mobility-shift and supershift for NF-κB

Nuclear protein extracts were obtained from cultured rat hepatocytes pretreated with TLCK (300 μM) (TL), TPCK (20 μM) (TP) or vehicle (—) for 30 min prior to adding TBH (50 μM for 12 h) or BSO (1 mM for 12 h) or pretreated for 12 h prior to adding DEM (0.2 mM for 20 min). Medium was changed after BSO and DEM treatment for another 6 h. Electrophoretic mobility-shift and supershift assays were performed as described in the Materials and methods section. Nuclear protein extracts from the control condition were used for supershift and competitive inhibition. The arrow-head indicates the specific NF-κB complex which was competitively blocked by 100× excess of unlabelled NF-κB probe (100× comp.).

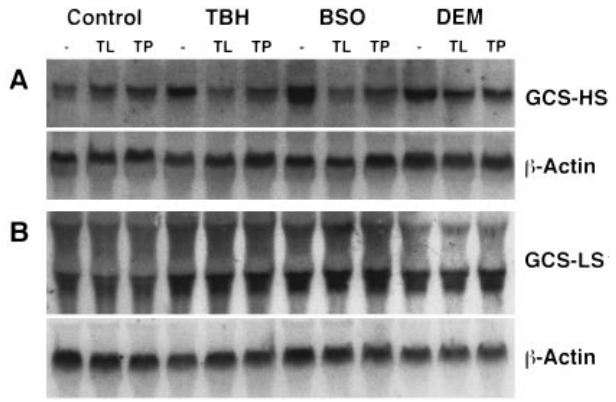

Figure 6 Effect of TLCK and TPCK on TBH-, BSO- and DEM-induced changes in GCS subunit mRNA levels

Total RNA (30 μg each lane) samples were obtained from cultured rat hepatocytes pretreated with TLCK (300 μM) (TL), TPCK (20 μM) (TP) or vehicle (—) for 30 min before adding TBH (50 μM for 12 h) or BSO (1 mM for 12 h), or were pretreated for 12 h before adding DEM (0.2 mM for 20 min). The medium was changed after BSO and DEM treatment for another 6 h. Northern-blot hybridization with GCS-HS, GCS-LS and β-actin cDNA probes were carried out as described in the Materials and methods section. A representative Northern blot from 3–6 separate experiments is shown for GCS-HS (**A**) and GCS-LS (**B**).

Table 2 Effect of TLCK and TPCK on TBH-, BSO- and DEM-induced changes in cell GSH and GCS-subunit mRNA levels in cultured rat hepatocytes

Cultured rat hepatocytes were pretreated with TLCK (300 μM), TPCK (20 μM) or vehicle for 30 min before the addition of TBH (50 μM for 12 h) or BSO (1 mM for 12 h) or were pretreated for 12 h before the addition of DEM (0.2 mM for 20 min). The medium was changed after BSO and DEM treatment for another 6 h. Northern-blot analysis was done using total RNA (30 μg) for both GCS-HS and GCS-LS. The same membranes were rehybridized with β-actin and the results were normalized to β-actin for comparison. The results are the means $\pm$ S.E. of 3–5 experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus TBH or BSO alone by ANOVA followed by Fisher's test.

		Percentage of control mRNA level	
Treatment	Cell GSH	GCS-HS	GCS-LS
TLCK	105 $\pm$ 15	100 $\pm$ 8	108 $\pm$ 13
TPCK	91 $\pm$ 12	104 $\pm$ 8	121 $\pm$ 6
TBH	78 $\pm$ 4*	181 $\pm$ 15*	180 $\pm$ 25*
TBH + TLCK	68 $\pm$ 9*	103 $\pm$ 6**	139 $\pm$ 27
TBH + TPCK	77 $\pm$ 13*	130 $\pm$ 5**	150 $\pm$ 27
BSO	3 $\pm$ 2*	195 $\pm$ 13*	205 $\pm$ 37*
BSO + TLCK	3 $\pm$ 1*	132 $\pm$ 18**	187 $\pm$ 19
BSO + TPCK	4 $\pm$ 2*	131 $\pm$ 28**	231 $\pm$ 20
DEM	110 $\pm$ 20	187 $\pm$ 8*	210 $\pm$ 6*
DEM + TLCK	105 $\pm$ 23	175 $\pm$ 19	179 $\pm$ 8
DEM + TPCK	95 $\pm$ 22	150 $\pm$ 5	197 $\pm$ 25

mRNA level (Figure 6B). Table 2 summarizes the densitometric results from 3–5 experiments. TLCK and TPCK did not significantly alter cell GSH in any of the treatment groups.

DISCUSSION

There is accumulating evidence in the literature that regulation of GCS is a major determinant of GSH homoeostasis. Many conditions are known to influence the steady-state mRNA level of GCS-HS [7–12,15]. These conditions fall into several categories. First is that of drug-resistant tumour cell lines [7–9], second is oxidative stress [10,11,26,27], third is anti-oxidants [12,27,28,29], fourth is the formation of Michael reaction acceptors (containing an electrophilic electron-deficient centre which is susceptible to nucleophilic attack) by treatment with DEM or phorone to produce GSH conjugates [12], fifth is growth-related such as hormones and low cell density [15], and sixth is the possibility of cell GSH itself [16,17,26]. Some of these conditions, namely oxidative stress, treatment with anti-oxidants and GSH conjugating agents, are believed to be capable of modulating gene expression via anti-oxidant responsive element. Such an element has been described in the promoter region of the human GCS-HS [30]. Recently, activation of NF-κB was shown to be the signalling mechanism for tumour necrosis factor-α- and interleukin-1β-induced stimulation of GCS-HS gene expression [26]. Since oxidative stress can activate NF-κB, it was important for us to examine whether NF-κB is involved in the signalling of oxidative-stress-induced increase in GCS subunit gene expression.

While many studies have examined gene regulation of the heavy subunit, no study to date has addressed gene regulation of the light subunit. The heavy subunit is active catalytically, but has a higher K_m for glutamate (18.2 versus 1.4 mM) and a lower K_i for GSH (1.8 versus 8.2 mM) when compared with the holoenzyme [6]. Thus it is possible, as suggested by Huang et al. [6], that the heavy subunit alone would not be active physiologically. This makes understanding the regulation of the light subunit all the more important. Gipp et al. [31] found no correlation between the steady-state mRNA levels of the two GCS subunits. Huang et al. [4] hinted that the liver may synthesize more light subunit than other tissues relative to the amount of heavy subunit although no data were provided. Thus factors that regulate their synthesis may be different. In fact, we found that hormones such as insulin and hydrocortisone and plating under low cell density influenced the steady-state mRNA level of the heavy subunit only. This further supports the notion that liver has more GCS-LS (or GCS-HS is limiting) since higher GCS activity occurred after treatments that increased only the heavy subunit mRNA and protein levels [13–15]. Treatment with DEM, BSO and TBH increased the steady-state mRNA level of both subunits by similar magnitudes (Table 1). Although TBH is considered as an antioxidant [29], it is capable of undergoing redox cycling which can generate superoxide and hydrogen peroxide [27]. Consistent with this is the fact that cell GSH fell at 12 h after treatment with TBH. Our results confirm previous reports of increased GCS-HS mRNA level after treatment of mice with DEM [12] or mouse hepatoma and rat lung epithelial L2 cells with TBH [27,29], albeit with slight differences in the timing of the peak effect.

Treatments with DEM, BSO and TBH have in common the capability of inducing oxidative stress and activation of NF-κB. When activation of NF-κB was blocked by TLCK and TPCK, the effect of BSO and TBH on stimulation of GCS-HS gene expression was blocked. Interestingly, TLCK and TPCK did not prevent to a significant degree the DEM-mediated increase in GCS-HS gene expression. This suggests different signalling pathways are involved for these treatments. Our data also suggest that NF-κB is not involved in the signalling of oxidative-stress-induced changes in GCS-LS gene expression. Although we cannot exclude other unknown effects of TLCK and TPCK which may have been responsible for blocking the increase in GCS-HS mRNA level by BSO and TBH, the fact that these treatments did not influence the basal or DEM-mediated increase in GCS-HS mRNA level or any of the treatments on GCS-LS mRNA level suggests blocking NF-κB activation was primarily responsible.

How activation of NF-κB influences GCS-HS gene expression will require additional studies.

The effect of BSO and the relationship between cell GSH and GCS subunit mRNA levels deserves comment. Our results show that significant changes in the mRNA levels did not occur until cell GSH was depleted to $< 10\,\%$ of controls. This might explain the reason for not detecting any BSO effect in the study by Borroz et al. [12], where hepatic GSH depletion was to only $30\,\%$ of controls. Other cell types and species may differ in this threshold level at which an increase in GCS subunit gene expression is observed. The mechanism of BSO may be via a change in the cell GSH itself (and thiol–disulphide ratio), induction of oxidative stress or both. Our data supports oxidative-stress-induced activation of NF-κB as the predominant signalling mechanism rather than cell GSH itself. This is because TLCK and TPCK blocked the BSO-induced increase in GCS-HS gene expression without affecting the cell GSH level of cells treated with BSO (Table 2). However, recent work of Urata et al. [26] suggests that cell GSH itself also modulates the expression of GCS-HS as an increase in the basal GCS-HS mRNA level was observed when GSH was increased by treatment of mouse endothelial cells with GSH ester. Whether this is also true of other cell types and how cell GSH level influences GCS-HS gene expression remains unknown.

In summary, we have shown that the two subunits of GCS are under different regulatory controls. Hormones and cell density influence the gene expression of the heavy subunit only, whereas treatment with DEM, BSO and TBH increase the gene expression of both subunits. Signalling through NF-κB is involved only in the oxidative-stress-mediated changes in the heavy subunit gene expression.

This work was supported by NIH grant DK-45334 and Professional Staff Association Grant #6-268-0-0, University of Southern California School of Medicine. Cultured rat hepatocytes were prepared by the Cell Culture Core of the University of Southern California Liver Disease Research Center (DK48522).

REFERENCES

1 Kaplowitz, N., Aw, T. Y. and Ookhtens, M. (1985) Annu. Rev. Pharmacol. Toxicol. **25**, 714–744
2 Richman, P. G. and Meister, A. (1975) J. Biol. Chem. **250**, 1422–1426
3 Yan, N. and Meister, A. (1990) J. Biol. Chem. **265**, 1588–1593
4 Huang, C., Anderson, M. E. and Meister, A. (1993) J. Biol. Chem. **268**, 20578–20583
5 Seelig, G. F., Simondsen, R. P. and Meister, A. (1984) J. Biol. Chem. **259**, 9345–9347
6 Huang, C., Chang, L., Anderson, M. E. and Meister, A. (1993) J. Biol. Chem. **268**, 19675–19680
7 Mulcahy, R. T., Untawale, S. and Gipp, J. J. (1994) Mol. Pharmacol. **46**, 909–914
8 Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C. and Anderson, M. E. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3070–3074
9 Mulcahy, R. T., Bailey, H. H. and Gipp, J. J. (1994) Cancer Chemother. Pharmacol. **34**, 67–71
10 Woods, J. S., Davis, H. A. and Baer, R. P. (1992) Arch. Biochem. Biophys. **296**, 350–353
11 Shi, M. M., Kugelman, A., Iwamoto, T., Tian, L. and Forman, H. J. (1994) J. Biol. Chem. **269**, 26512–26517
12 Borroz, K. I., Buetler, T. M. and Eaton, D. L. (1994) Toxicol. Appl. Pharmacol. **126**, 150–155
13 Lu, S. C., Ge, J., Kuhlenkamp, J. and Kaplowitz, N. (1992) J. Clin. Invest. **90**, 524–532
14 Lu, S. C. and Ge, J. (1992) Am. J. Physiol. **263**, C1181–1189
15 Cai, J., Sun, W. and Lu, S. C. (1995) Mol. Pharmacol. **48**, 212–218
16 Yao K. S., Godwin, A. K. Ozols, R. F., Hamilton, T. C. and O'Dwyer, P. J. (1993) Cancer Res. **53**, 3662–3666
17 Walsh, A. C., Michaud, S. G., Malossi, J. A. and Lawrence, D. A. (1995) Toxicol. Appl. Pharmacol. **133**, 249–261
18 Moldeus, P., Hogberg, J. and Orrenius, S. (1978) Methods Enzymol. **51**, 60–70
19 Tietze, F. (1969) Anal. Biochem. **27**, 502–522
20 Runnegar, M., Kong, S., Zhong, Y. and Lu, S. C. (1995) Biochem. Pharmacol. **49**, 219–225
21 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23 Baeuerle, P. A. and Baltimore, D. (1989) Genes Dev. **3**, 1689–1698
24 Fitzgerald, M. J., Webber, E. M., Donovan, J. R. and Fausto, N. (1995) Cell Growth Differ. **6**, 417–427
25 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Nucleic Acids Res. **17**, 6419
26 Urata, Y., Yamamoto, H., Goto, S., Tsushima, H., Akazawa, S., Yamashita, S., Nagataki, S. and Kondo, T. (1996) J. Biol. Chem. **271**, 15146–15152
27 Liu, R. M., Hu, H., Robison, T. W. and Forman, H. J. (1996) Am. J. Respir. Cell Mol. Biol. **14**, 186–191
28 Eaton, D. L. and Hamel, D. M. (1994) Toxicol. Appl. Pharmacol. **126**, 145–149
29 Liu, R. M., Vasiliou, V., Zhu, H., Duh, J. L., Tabor, M. W., Puga, A., Nebert, D. W., Sainsbury, M. and Shertzer, H. G. (1994) Carcinogenesis **15**, 2347–2352
30 Mulcahy, R. T. and Gipp, J. J. (1995) Biochem. Biophys Res. Comm. **209**, 227–233
31 Gipp, J. J., Bailey, H. H. and Mulcahy, R. T. (1995) Biochem. Biophys. Res. Commun. **206**, 584–589

Received 27 January 1997/24 March 1997; accepted 23 April 1997