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Hormonal and Cell Density Regulation of Hepatic γ-Glutamylcysteine Synthetase Gene Expression

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SUMMARY

We previously reported that the activity of γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in GSH synthesis, is under both hormonal and cell density regulation in cultured rat hepatocytes. Specifically, the addition of insulin or hydrocortisone to culture media or the lowering of the initial plating cell density increased cell GSH by increasing the activity of GCS. In the present study, we examined the molecular mechanism of these effects. To determine whether the increase in GCS activity is associated with an increase in GCS heavy subunit (GCS-HS) mRNA expression, the steady state mRNA levels of GCS-HS were examined with the use of Northern blots. After 24-hr treatment of high density (0.6×10^5 cells/cm^2) cultured rat hepatocytes with insulin (1 μg/ml) or hydrocortisone (50 nM), the steady state GCS-HS mRNA level increased by ~1–2 fold. When the plating density was decreased to 0.1×10^5 cells/cm^2, the steady state GCS-HS mRNA level also increased by 1–2 fold 24 hr later. An increase in the steady state GCS-HS mRNA level was found within 4 hr of either hormonal treatment or cell density manipulation. The increase in steady state GCS-HS mRNA level resulted from increased gene transcription, as the transcriptional rates of GCS-HS after hormonal or cell density manipulation were increased by 2–3-fold, whereas the rates of GCS-HS mRNA degradation remained unchanged. Western blotting confirmed the increase in GCS-HS protein level after hormone treatment or lowering of plating cell density. When examined *in vivo*, the steady state GCS-HS mRNA level decreased by 50% in a rat in which diabetes had been induced with streptozotocin for 1 week; this was prevented with insulin replacement. In summary, GCS-HS gene expression is under both hormonal and cell density regulation.

GSH is a tripeptide, γ-glutamylcysteinylglycine, that is synthesized by virtually all mammalian cells. The concentration of GSH is particularly high in the liver, which plays a central role in the interorgan homeostasis of GSH (1, 2). Hepatic GSH plays a vital defensive role against toxins and free radicals and is involved in the storage and transfer of cysteine (1). The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic 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 catalyzed by GCS, which is regulated physiologically through feedback competitive inhibition by GSH and the availability of cysteine (1, 3). The GCS enzyme is composed of a heavy subunit (M_r ~73,000) and a light subunit (M_r ~28,000) that are connected by disulfide bonds. The two subunits are encoded for by different genes, which have been cloned (4, 5). The heavy subunit exhibits all of the catalytic activity of the isolated enzyme as well as feedback inhibition by GSH (6). The light subunit is enzymatically inactive but plays an important regulatory function by lowering the K_m of GCS for glutamate and raising the K_i for GSH (5, 7). Thus, the holoenzyme is catalytically more efficient and less subject to inhibition by GSH than the heavy subunit. In many conditions (i.e., drug-resistant tumor cell lines, treatment with methyl mercury hydroxide) where GCS activity is increased, there also is an increase in the GCS heavy subunit (GCS-HS) mRNA level (8–12). Thus, regulation of the GCS-HS gene expression appears critical for GSH homeostasis.

We have been interested in studying the regulation of GCS. We and others have reported that GSH synthesis was inhibited by hormone-mediated activation of various signal transduction pathways, (13, 14) leading to an acute inhibition of GCS activity (13). We have also shown in primary cultures of rat hepatocytes that the activity of GCS can be induced by Ins (1 μg/ml) or HC (50 nM) treatment (15) and by lowering the initial plating cell density (16). These effects were seen

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ABBREVIATIONS: GSH, reduced glutathione; LD, low plating cell density; HD, high plating cell density; GCS, γ-glutamylcysteine synthetase; GCS-HS, γ-glutamylcysteine synthetase heavy subunit; HC, hydrocortisone; Ins, insulin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NIB, nuclear isolation buffer; NFB, nuclear freezing buffer; TBST, 10 mM Tris, pH 8.0, 150 mM NaCl, containing 0.05% Tween 20; bp, base pair(s); SDS; sodium dodecyl sulfate; SSC; standard saline citrate.

after a lag of 6 hr in the former and 2 hr in the latter case. In both cases, the induction of GCS activity was blocked by either cycloheximide or actinomycin D treatment. We also assessed the physiological significance of the hormone effect using *in vivo* models such as insulin-deficient diabetic or adrenalectomized rats. Both exhibited lower hepatic GSH levels and GCS activity, which were prevented with hormone replacement (15). In the case of cell density, lowering the initial plating density effectively shifts adult rat hepatocytes from G_o to G_1 phase of the cell cycle, analogous to liver regeneration after partial hepatectomy or after cell death (17). Consistent with our findings in cultured rat hepatocytes, transitory elevation in cellular GSH was reported in regenerating rat liver before the increase in DNA synthesis and recovery from liver necrosis (18, 19). Thus, our previous data suggest that hepatic GCS gene expression may be regulated by hormones and changes with cell cycle from G_o to G_1 phase. The aim of the present study was to test this hypothesis by 1) examining whether induction of GCS activity by hormones and lowering of initial plating cell density is associated with an increase in the steady state GCS-HS mRNA levels and 2) determining if GCS-HS mRNA level is increased, whether that is a result of increased transcriptional rate, decreased mRNA breakdown, or both.

Experimental Procedures

Materials. GSH, collagenase (type IV), bovine serum albumin, L-methionine, NADPH, 5,5′-dithiobis (2-nitrobenzoic acid), sodium EDTA, GSH reductase, Ins, HC, streptozotocin, FBS, CTP, GTP, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). DME/F12 medium was purchased from Irvine Scientific (Irvine, CA). Ins (NPH-Ilettin, beef/pork) was obtained from Eli Lilly and Co. (Indianapolis, IN). ^{32}P-dCTP (3000 Ci/mmol) and ^{32}P-dUTP (3000 Ci/mmol) were purchased from New England Nuclear-DuPont (Boston, MA). Total RNA isolation kit was obtained from Promega (Madison, WI). 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), weighing 260–320 g were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO) and water *ad libitum*.

Cell culture preparation. Isolation of hepatocytes was done aseptically according to the method of Moldeus *et al.* (20). Initial cell viability was >90% as determined by 0.2% trypan blue exclusion. The plating medium was DME/F12 and high glucose (3151 mg/L) supplemented with excess methionine (1 mM) and 10% FBS. HD (0.6×10^5 cells/cm^2) was used to examine the effect of hormones. LD was 0.1×10^5 cells/cm^2. Cells were plated on either 60 × 15-mm or 100 × 15-mm dishes precoated with rat tail collagen using 5 or 10 ml plating medium, respectively, and incubated at 37° in 5% CO_2-95% air. Two hours after plating, medium was changed to remove dead, unattached cells and to omit FBS. Cell attachment averaged ~60%. At this medium change, either Ins (1 μg/ml), HC (50 nM), or vehicle was added for 4 or 24 hr.

Induction of diabetes. The protocol followed was as described previously (15). Rats were treated with streptozotocin (80 mg/ml in 0.05 M citric acid-Na_2HPO_4 mixture, pH 4.2) intraperitoneally (60 mg/kg). Control rats received vehicle. Moderate diabetes was achieved in the majority of animals within 24 hr as determined by measuring blood glucose with the use of Chemstrip bG with Accu-Check II (Boehringer Mannheim Diagnostics). Only animals with a glucose level of >350 mg/dl 1 day after streptozotocin treatment were considered to be insulin deficient and were included in the study. To study the effect of insulin, 1 day after streptozotocin injection diabetic rats were randomized to receive Ins (NPH, Eli Lilly) at a dosage of 1–2 U at approximately 9:00 a.m. and 2–3 U at approximately 4:30 p.m. subcutaneously for 1 week, which maintained the glucose level at an average of approximately 200 mg/dl. Control and untreated diabetic rats received saline subcutaneously.

RNA isolation and Northern blot analysis. RNA was isolated from primary cultures of rat hepatocytes and from frozen liver biopsies according to the method of Chomczynski and Sacchi (21). Total RNA was isolated from cultured cells 4 and 24 hr after the addition of hormones or vehicle. The GCS-HS cDNA probe in pCR II plasmid was kindly provided by Dr. James S. Woods (Department of Environmental Health, School of Public Health and Community Medicine, University of Washington, Seattle, WA) and was composed of a 390-bp fragment corresponding to nucleotides 79–468 of the published rat kidney GCS-HS sequence (4) and labeled with [^{32}P]dCTP with the use of a random-primer kit (Primer-It II Kit; Stratagene, La Jolla, CA).

Total RNA (20 μg) obtained from liver of a single animal or pooled from culture plates of the same condition (HD, Ins, HC, or LD) derived from one animal was separated on a 1% agarose gel with 6.7% formaldehyde. RNA was then blotted overnight onto a nylon membrane by capillary transfer in 20 × SSC (1 × SSC: 15 mM sodium citrate, 0.15 M NaCl, pH 7.0). The filters were exposed to a source of ultraviolet irradiation (UV Cross-Linker, FB-UVXL-1000, Fisher Biotech), prehybridized for 1–2 hr at 65° in Rapid-Hybridization buffer (Amersham Life Science, Arlington Heights, IL), and hybridized for 4 hr at 65° with the labeled GCS-HS cDNA probe (2×10^6 cpm/ml) in the same buffer. Next, the filters were washed three times (15 min each) with 2 × SSC/0.1% SDS at room temperature and three times (30 min each) with 0.1 × SSC/0.1% SDS at 65°. The filters were autoradiographed for 12–72 hr onto Hyperfilm (Amersham Corp., Arlington Heights, IL) with image-intensifying screens at −70°. To ensure equal loading of RNA samples, the same membrane was rehybridized with ^{32}P-labeled human β-actin cDNA probe (Clontech, Palo Alto, CA). Autoradiography and densitometry (2222–020 Ultra Scan XL laser densitometer with software 3.0; LKB) were used to quantify relative RNA.

Nuclear run-on transcription assay. Nuclei were isolated from cultured hepatocytes after treatment of cells with hormones or were plated under varying densities for 4 hr according to the procedure described by Greenberg *et al.* (22) with slight modifications. Cells from several plates were washed with PBS and harvested with trypsin-EDTA (0.05% and 0.02%, respectively). The cell suspension was pelleted at 500 × *g* for 5 min (Beckman GPR centrifuge). The pellet was resuspended in ice-cold NIB (10 mM Tris·HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 M sucrose, and 0.5%, v/v, Nonidet P-40), incubated for 10 min on ice, and centrifuged at 1000 × *g* at 0° for 5 min. The nuclear pellet was washed two or three times in ice-cold NIB by gentle resuspension and centrifugation as described. The supernatant was discarded, and 2×10^7 nuclei (determined with a hemocytometer) were resuspended in 50 μl NFB (50 mM Tris·HCl, pH 8.0, 50%, v/v, glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA), snap-frozen with liquid N_2, and kept at −80° or used immediately.

The procedures for labeling and isolating the actively transcribed RNA were adapted from Lee *et al.* (19) and Kren *et al.* (23). The same number of nuclei (2×10^7) obtained under different experimental conditions (hormone treatment and varying densities) were incubated in 25% glycerol, 25 mM Tris·HCl, pH 8.0, 140 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.05 mM EDTA, 1 mM dithiothreitol, 100 U placental ribonuclease inhibitor, 0.25 mM CTP, 0.25 mM GTP, 0.25 mM ATP, and 250 μCi [^{32}P]UTP (3000 Ci/mmol) in a final volume of 100 μl for 30 min at 30°. The reaction was stopped by adding 6 μl of a solution of 5 M NaCl, and the DNA was digested by adding 12 μl of a 1 mg/ml solution of RQ1 DNase (Promega Corp, Madison, WI) and incubating for 15 min at 30°. RNA was extracted twice with phenol/chloroform (1:1, v/v) and once with ether and precipitated with 0.1 volume of 2.5 M sodium acetate and 3 volumes of absolute ethanol.

Precipitation was repeated twice, and the pellet was dried under vacuum and resuspended in DEPC-treated H_2O. The amount of labeling was determined by counting 2-μl aliquots of each sample with the use of a scintillation counter (Beckman Scintillation counter LS6000TA) and was nearly identical under different experimental conditions.

Hybridization of nuclear labeled RNAs to plasmid DNA was done by first denaturing plasmid DNAs containing GCS-HS or β-actin cDNA insert (2 μg) for 10 min in a boiling bath and binding to nylon filters with a slot blot apparatus (Bio-Dot SF, Bio-Rad, Hercules, CA). The filter was air-dried, washed with 6 × SSC for 15 min at room temperature, and exposed to UV Crosslinker as described above. Prehybridization was done at 65° in 2 ml Rapid-Hybridization buffer. After 2 hr, the filters were hybridized for 12–24 hr at 65° in 1 ml of the same buffer containing identical amounts of labeled RNAs (1–10 × 10^7 cpm) obtained from the different run-on transcriptions. After hybridization, the filters were washed twice (15 min each) with 2 × SSC-0.1% SDS at room temperature and twice (15 min each) with 0.2 × SSC-0.01% SDS at 65° and subjected to autoradiography. Quantification was done by scanning densitometry of the autoradiograms as above. The pCR II vector served as a negative control that was subtracted as background from the densitometry readings. Results were normalized to β-actin.

Rate of degradation of GCS-HS mRNA. To assess the stability of GCS-HS mRNA after various treatments, the protocol used by Rosewicz *et al.* (24) was adapted. Cultured cells were treated with HC or Ins or plated under LD, and 24 hr later RNA was isolated as described at 0, 60, 120, and 180 min after the addition of actinomycin D (10 μg/ml). The amount of GCS-HS mRNA was quantified by using the ^{32}P-labeled GCS-HS cDNA probe in slot blot analysis as described (24). Results were normalized to β-actin and expressed as a percent of the initial GCS-HS mRNA concentration.

Measurement of GSH. Cultured cells plated on collagen-precoated dishes were detached by trypsin-EDTA (0.05% and 0.02%, respectively). Cells were treated with 10% trichloroacetic acid to extract cellular GSH. The mixture was centrifuged at 13,000 × g in a microfuge (Beckman) for 1 min to remove the denatured proteins, and GSH was measured in the supernatant by the recycling method of Tietze (25). Cell number was determined by Coulter counter.

Western blot of GCS-HS. Rabbit polyclonal antibody against a synthetic peptide (TVEDNMRKRRKEA), which corresponds to amino acid residues 119–131 of rat kidney GCS-HS (4), was used for Western blot analysis. The peptide was chosen according to the Hopp and Woods Hydrophilicity Scale (26). Both peptide synthesis and antibody generation were carried out with the use of Multiple Peptide Systems (San Diego, CA).

Cultured cells were treated with HC (50 nM) or Ins (1 μg/ml) or plated under LD, and 24 hr later cells were scraped off. Extracts were prepared by, first, homogenization with homogenizing buffer (250 mM sucrose, 10 mM phosphate buffer, pH 7.4) with the use of 1-ml tissue grinder (Reacti-Ware; Pierce) and, second, centrifugation at 13,000 × g in a microfuge for 10 min. The supernatant was used for Western blotting. For comparison, rat kidney homogenate was included in the Western blots. Protein concentration was determined by the Bio-Rad protein assay and was 5–6 mg/ml. Cell extracts or kidney homogenates containing 20–50 μg of protein were solubilized in equal volumes of sample buffer (285 mM Tris, pH 6.8, 30% glycerol, 6% SDS, 1.5% 2-mercaptoethanol, and 0.01% bromphenol blue), and subjected to SDS-10% PAGE (27), and electrotransferred to nitrocellulose membranes with the use of Semidry Transfer Cell (Bio-Rad) (28). The nitrocellulose membranes were subsequently subjected to the Amplified Alkaline Phosphatase Immun-Blot Assay according to procedures described in the kit (170–6412, Bio-Rad). The first antibody was rabbit anti-rat kidney GCS-HS peptide preimmune or postimmune serum diluted 1:250 in TBST.

Statistical analysis. For cultured cells, each cell preparation was derived from one animal, and multiple plates were used for each condition. Multiple plates were pooled for RNA and nuclei isolation. Each Northern or slot blot was ran using pooled RNA sample from the same treatment condition derived from one cell preparation or an individual animal. The quantification was done by autoradiography and densitometry. After normalization to β-actin, results of multiple Northern or slot blots were compared by either paired Student's t test (two comparisons) or analysis of variance (more than two comparisons) followed by Fisher's test. Two-tailed t tests were used unless otherwise noted.

Results

Effect of Hormones and Cell Density on GCS-HS mRNA Levels of Cultured Hepatocytes

Table 1 is a summary of the effect of hormone treatment and plating cell density on cell GSH in the cell preparations that were used for Northern blot analysis. Similar to our previous observations (15, 16), cell GSH level increased significantly after 24-hr treatment of cultured rat hepatocytes with either HC (50 nM) or Ins (1 μg/ml) or by simply lowering the plating cell density. Fig. 1 shows a representative Northern blot from these experiments on steady state GCS-HS mRNA level. Changes in relative expression of mRNA were normalized to β-actin. The increase in GCS-HS mRNA level was evident 4 hr after the addition of the hormones. As shown in Table 2, after 24 hr of either HC or Ins treatment, the steady state GCS-HS mRNA level increased by 187% and 123%, respectively.

Fig. 2 shows the effect of varying plating cell densities on steady state GCS-HS mRNA level. Fig. 2 is a representative Northern blot from four experiments. The results of four experiments are summarized in Table 2. Twenty-four hours after plating, steady state GCS-HS mRNA level increased by 146% in cells plated under LD. The increase in GCS-HS mRNA was also evident at 4 hr (data not shown).

Effect of Diabetes Mellitus and Ins Replacement on Steady State GCS-HS mRNA Level

We previously reported that streptozotocin-induced diabetic rats exhibited lower hepatic GSH and GCS activities, which were prevented with Ins replacement (15). To determine whether changes in GCS-HS mRNA expression were responsible for this, we used Northern blot analysis to examine GCS-HS mRNA levels in livers of control, diabetic, and Ins-treated animals. Fig. 3 is a representative Northern blot from these animals. The results for all animals are shown in Table 2. Untreated diabetic animals had lower steady state

TABLE 1

Effects of hormones and plating cell density on cell GSH levels of cultured rat hepatocytes

Condition	Cell GSH
	nmol/10^6 cells
HD	
Control	41.2 ± 3.9
HC	70.5 ± 12.7*
Ins	66.5 ± 8.5*
LD	
Control	71 ± 5.9*

Results are expressed as mean ± standard error from four or five cell preparations. Cultured hepatocytes were plated at HD (0.6 × 10^5/cm^2) and LD (0.1 × 10^5/cm^2) for 24 hr. Hormonal effect was assessed by adding HC (50 nM) or Ins (1 μg/ml) to medium for 24 hr. Cell GSH level was determined by the method of Tietze (25).

* $p < 0.05$ vs. HC control by analysis of variance.

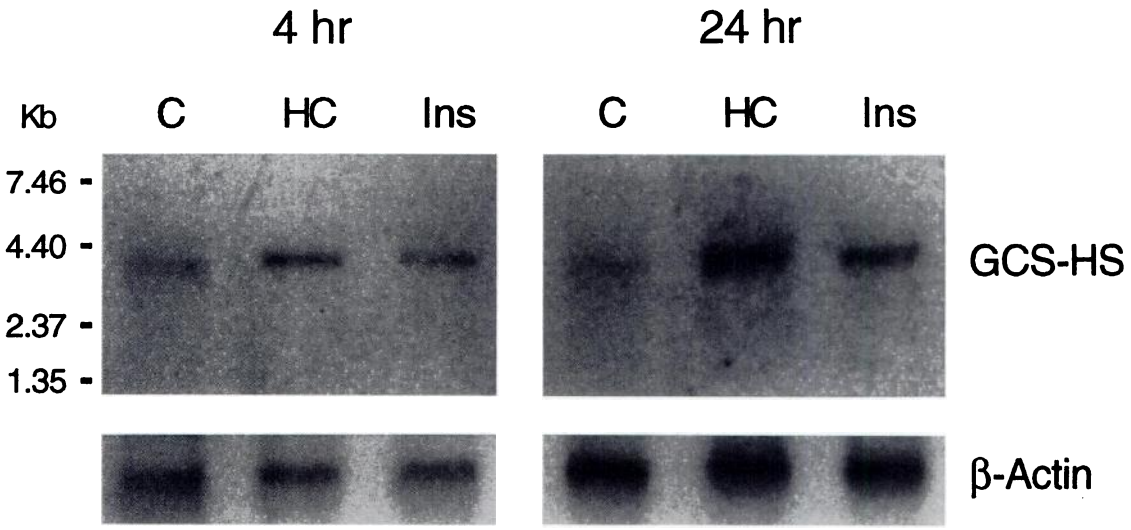

Fig. 1. Effects of HC (50 nM) and Ins (1 μg/ml) on steady state GCS-HS mRNA level in cultured rat hepatocytes. RNA (20 μg/lane) samples obtained from cultured rat hepatocytes treated with HC or Ins or vehicle control (*C*) for 4 or 24 hr were analyzed by Northern blot hybridization with a ^{32}P-labeled GCS-HS cDNA probe as described in Methods. The same membrane was then rehybridized with ^{32}P-labeled human β-actin cDNA probe. Molecular size markers, GCS-HS (~3.7 kb), and β-actin are as indicated. Relative RNA amounts were normalized to β-actin, and quantification was done with autoradiography and densitometry. Shown is representative Northern blot.

TABLE 2

Effects of hormones and plating cell density on GCS-HS mRNA level and GCS-HS transcriptional rate

Condition	% of Control
GCS-HS mRNA level	
Cultured rat hepatocytes	
HC	287 ± 63*
Ins	223 ± 14*
LD	246 ± 35*
Diabetic and Ins-treated animals	
Untreated diabetic animals	52 ± 5*
Ins-treated	93 ± 8
GCS-HS transcription rate	
HC	328 ± 37*
Ins	420 ± 77*
LD	320 ± 67*

Results are expressed as mean percent of control ± standard error from three to five cell preparations. Cultured hepatocytes were plated at HD (0.6×10^5/cm^2) or LD (0.1×10^5/cm^2) for 24 hr. Hormonal effect was assessed by adding HC (50 nM) or Ins (1 μg/ml) to medium of HD cells for 24 hr. Effect of streptozotocin-induced diabetes and insulin replacement was assessed after 1 week of induction. Steady state GCS-HS mRNA level and transcriptional rate were measured as described in Methods.

* $p < 0.05$ vs. control by analysis of variance followed by Fisher's test.

GCS-HS mRNA levels (52% of control), which were prevented with Ins replacement.

Mechanism of Increase in Steady State GCS-HS mRNA Level in Cultured Rat Hepatocytes

Transcriptional rate of GCS-HS. Steady state mRNA levels represent a balance between *de novo* synthesis and degradation of mRNA molecules. To determine whether the increase in steady state GCS-HS mRNA levels resulted from increased gene transcription, we used the nuclear run-on transcriptional assay. We chose to examine the effects of hormones and LD on GCS-HS gene transcription at 4 hr because by then the steady state GCS-HS mRNA level had increased. Fig. 4 is a representative blot from one of three experiments. All three conditions (i.e., HC, Ins, and LD) led to an increase in the level of newly transcribed GCS-HS mRNA. These treatments did not alter the transcriptional rate of β-actin (data not shown). After subtracting the vector

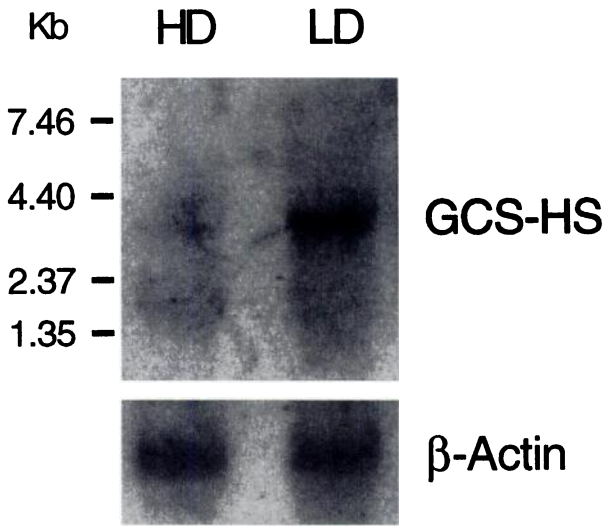

Fig. 2. Effects of varying initial cell-plating densities on steady state GCS-HS mRNA level in cultured rat hepatocytes. RNA (20 μg/lane) samples obtained from cultured rat hepatocytes plated under HD (0.6×10^5 cells/cm^2) or LD (0.1×10^5 cells/cm^2) for 24 hr were analyzed by Northern blot hybridization with a ^{32}P-labeled GCS-HS cDNA probe as described in Methods. The same membrane was then rehybridized with ^{32}P-labeled human β-actin cDNA probe. Molecular size markers, GCS-HS (~3.7 kb), and β-actin are as indicated. Relative RNA amounts were normalized to β-actin, and quantification was done using autoradiography and densitometry. Shown is representative Northern blot.

as background and normalizing to β-actin, both hormones and LD had a 2--3-fold increase in the level of newly transcribed GCS-HS mRNA. Table 2 provides a quantitative summary of the results of these three experiments.

Stability of GCS-HS mRNA. To determine whether hormonal treatment or lowering of the initial plating cell density altered the stability of GCS-HS mRNA, cells were first treated with Ins (1 μg/ml) or HC (50 nM) or plated under LD for 24 hr. Then GCS-HS mRNA was quantified at various times after the addition of actinomycin D (10 μg/ml). The level of β-actin mRNA remained constant over 180 min, which is consistent with its reported long half-life of >12 hr

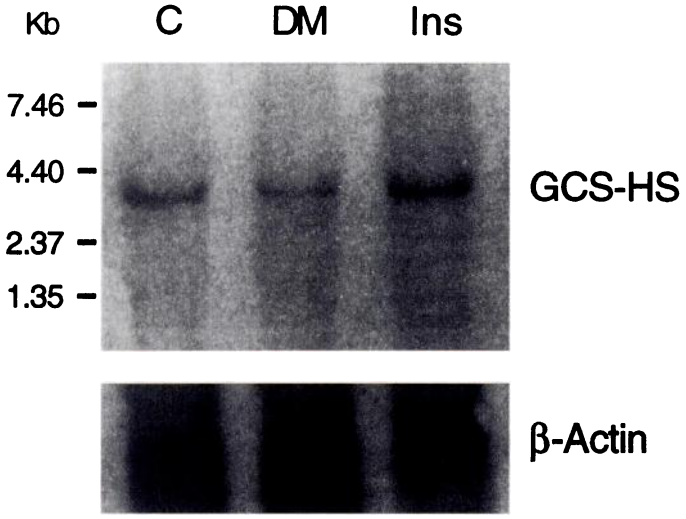

Fig. 3. Effects of streptozotocin-induced diabetes (*DM*) and Ins replacement on steady state liver GCS-HS mRNA level. RNA (20 μg/lane) samples isolated from livers of control (*C*), 1-week DM, and Ins-treated diabetic rats were analyzed by Northern blot hybridization with a ^{32}P-labeled GCS-HS cDNA probe as described in Methods. Same membrane was then rehybridized with ^{32}P-labeled human β-actin cDNA probe. Molecular size markers, GCS-HS (~3.7 kb), and β-actin are as indicated. Relative RNA amounts were normalized to β-actin, and quantification was done using autoradiography and densitometry. Shown is representative Northern blot.

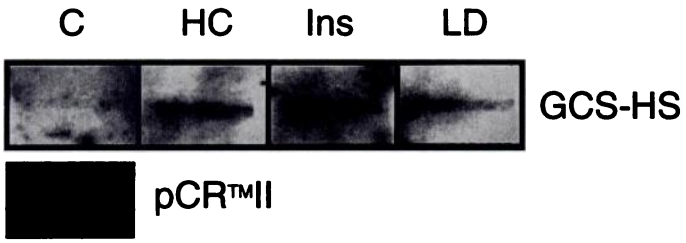

Fig. 4. Effect of HC, Ins and varying initial cell-plating densities on GCS-HS gene transcription in cultured rat hepatocytes. Cultured cells were treated with HC (50 nM) or Ins (1 μg/ml) or plated under LD as described for 4 hr and had nuclei isolated for nuclear run-on transcription assay performed as described in Methods. Representative slot blot is shown; the plasmid vector, pCR II, served as a negative control.

(24). As Fig. 5 shows, the half-life of GCS-HS mRNA was not altered by any of the treatments. The estimated half-life of GCS-HS mRNA was approximately 5 hr. Thus, an increase in GCS-HS gene transcription was responsible for the increase in the steady state GCS-HS mRNA level.

Western Blot Analysis of GCS-HS

To conclusively demonstrate that the increase in GCS activity observed previously with Ins and HD treatment or lowered plating cell density was due to an increase in the GCS-HS protein level, we performed semiquantification of GCS-HS protein by Western blot analysis with cell extracts of cultured rat hepatocytes treated with Ins (1 μg/ml) or HC (50 nM) or plated under LD for 24 hr. Fig. 6 is a representative blot of four separate cell preparations. The bands at 73 kDa in cell extracts and rat kidney correspond to the molecular mass of rat GCS-HS (4). Specificity was confirmed by using preimmune and postimmune sera. As predicted, hormone treatment and lowering of plating cell density resulted in increased GCS-HS protein level.

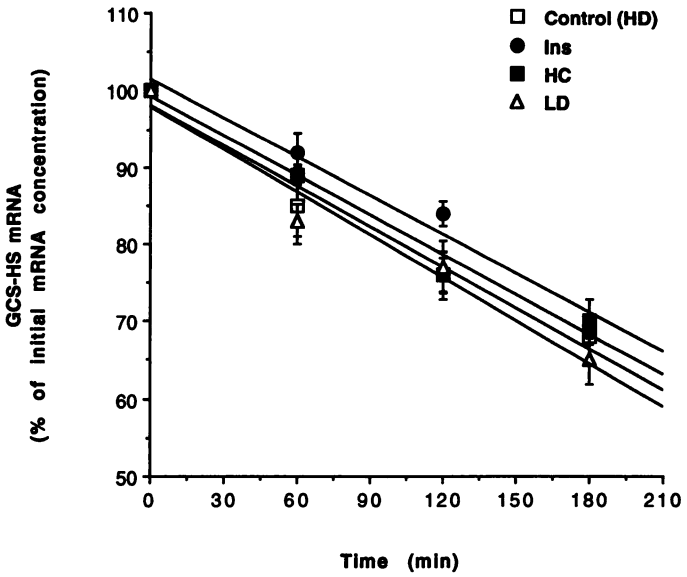

Fig. 5. Effect of Ins, HC, and varying initial cell plating densities on stability of GCS-HS mRNA. Cells were plated under HD or LD as described in Methods, and Ins (1 μg/ml) or HC (50 nM) was added to HD cells. After 24 hr, total RNA was isolated at 0, 60, 120, and 180 min after the addition of actinomycin D (10 μg/ml). GCS-HS mRNA was quantified using slot blot analysis, normalized to β-actin after autoradiography and densitometry. Results are expressed as mean percent ± standard error of initial GCS-HS mRNA level for each condition from three cell preparations. Rate of fall in GCS-HS mRNA as determined by linear regression did not differ among these conditions (control [HD] = 0.175%, Ins = 0.168%, HC = 0.172%, and LD = 0.185% of initial GCS-HS mRNA/min).

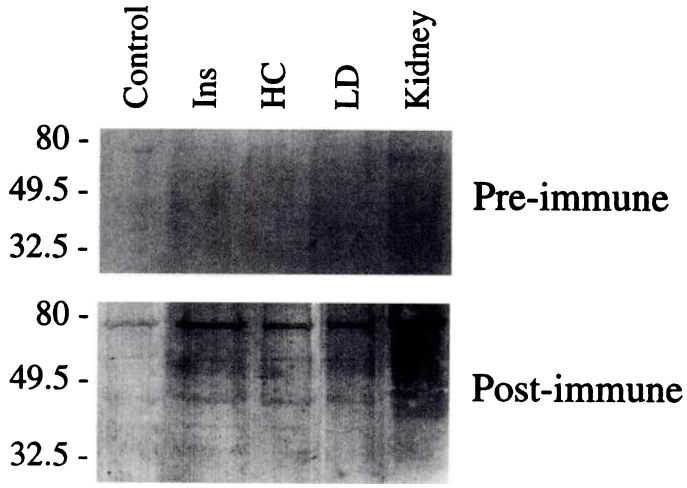

Fig. 6. Effect of Ins, HC, and varying initial cell plating densities on GCS-HS protein level. Cells were plated under HD or LD as described in Methods, and Ins (1 μg/ml) or HC (50 nM) was added to HD cells. After 24 hr, cell extracts (50 μg of protein per lane) were subjected to SDS-PAGE followed by immunoblotting with preimmune and postimmune sera. Rat kidney homogenates (20 μg/lane) were included for comparison. *Left*, Molecular weight markers (in kDa).

Discussion

Intracellular GSH content is influenced by several factors: 1) the availability of sulfur amino acid precursors, 2) the activity of GCS, 3) the rate of GSH efflux, and 4) intracellular use of GSH. An increased steady state GCS-HS mRNA level has been found in many conditions where GCS activity is induced (8–12, 15, 16). Specific examples include drug-resis-

tant tumor cell lines (8–10) and oxidative stress (11, 12). Recently, transcriptional upregulation of the GCS-HS was described in melphalan-resistant human prostate carcinoma cells (8). We first reported that hepatic GCS activity is also under hormonal and cell density regulation (15, 16). In the present study, we examined the involvement of GCS-HS.

The results of the present study confirmed our previous findings that both HC and Ins as well as lowering of the initial plating cell density significantly raised cell GSH by 60–70%. This was associated with an increase in the steady state GCS-HS mRNA level, which resulted from an increase in GCS-HS gene transcription without affecting the degradation of GCS-HS mRNA. Furthermore, Western blot analysis confirmed the increase in GCS-HS protein level. The present study was focused only on the heavy subunit of GCS. Because the light subunit of GCS plays an important regulatory role in overall GCS activity, it is also important to examine the effects of hormones and cell density on the light subunit gene expression. Research involving such characterizations are in progress.

Both Ins and glucocorticoids are well known for having important regulatory effects on the expression of many genes (29, 30). Glucocorticoids are known to bind to cytosolic glucocorticoid receptors avidly but noncovalently (29). This process results in activation or transformation of the hormone-receptor complex into a DNA-binding protein, which modulates the transcription of specific genes by binding to target sequences referred to as glucocorticoid-responsive, or regulatory, elements. The mechanism of Ins is much more complex as multiple cytoplasmic mediators may be involved. Much remains unknown regarding the precise mechanism of how Ins modulates the expression of many genes. Both stimulatory and inhibitory effects on gene transcription have been described (30). Similar to our observations with GCS-HS, the effects of Ins on steady state mRNA levels of many of the Ins-regulated genes usually are only 2–3-fold in magnitude (30). It is of importance that the effect of Ins on GCS-HS gene expression was observed with both cultured cells and in Ins-deficient diabetic rats. This strongly supports a direct effect of Ins activity on GCS-HS gene expression. In contrast to glucocorticoids, no sequence-specific binding to DNA by Ins or the Ins receptor has been described. Clearly, much work will be required to elucidate the exact molecular mechanism by which these hormones regulate the gene expression of GCS-HS.

In many cell types, an increased GSH level has been associated with an early proliferative response, which is essential for the cell to enter the S phase (31–33). Plating mature hepatocytes under LD effectively shifts cells from phase G_0 to G_1 of the cell cycle (17). We reported that an inverse relationship exists between cell GSH and plating cell density (16). This occurred in the absence of hormones or any growth factors and was mediated by both an increase in the GCS activity and the availability of cysteine. Interestingly, the increase in GCS activity under LD was blocked when co-cultured with HD cells, suggesting that soluble factor(s) were responsible for the effect. The present study showed that the increase in GCS activity under LD was mediated by an increase in GCS-HS gene transcription. Future work will focus on defining the soluble factor(s) responsible for this effect.

In summary, we showed for the first time that the gene for GCS-HS can be transcriptionally upregulated by hormones and lowering of plating cell density. The pathophysiological implications of these findings remain to be defined.

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