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Glutathione and bile acid synthesis. Effect of GSH content of HepG2 cells on the activity and mRNA levels of cholesterol 7α-hydroxylase

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Abstract—Cholesterol 7α-hydroxylase (CH-7α) activity in HepG2 cells depleted of glutathione (GSH) was reduced significantly ($P < 0.05$) compared to that in untreated controls. Northern blot analysis of poly A^+ mRNA isolated from GSH-depleted and control HepG2 cells showed that there was a reduction in mRNA for CH-7α in treated HepG2 cells that was commensurate with the reduction in CH-7α activity. The fact that total RNA, rRNA, and mRNA for β fibrinogen were unaltered by the depletion of GSH suggests that the change in steady-state CH-7α mRNA content is specifically sensitive to GSH content. This observation represents the first demonstration, for human liver cells, that there is an interaction between GSH levels and the regulation of CH-7α mRNA levels.

Hepatic cholesterol 7α-hydroxylase (CH-7α*, EC 1.14.13.17) is the rate-limiting enzyme in the catabolism of cholesterol to bile acid [1]. In addition to the well-documented diurnal rhythm in the activity of CH-7α [1], several other factors are also believed to influence the activity of CH-7α [2–5]. This laboratory has been involved in studying the effect of hepatic glutathione (GSH) content on CH-7α activity [6–9]. The data support the thesis that CH-7α activity is sensitive to hepatic GSH content; however, the nature of the relationship is not known. Recently, several laboratories have been successful in purifying this enzyme and obtaining the cDNA [10–12]. As a result, it has been possible to study the molecular basis for the regulation of this enzyme. Additionally, the availability of a fairly well-differentiated human hepatoma cell line, HepG2 [13], makes it possible to study regulatory aspects of CH-7α under well-controlled conditions. Thus, the objective of the present report was to study the effect of GSH depletion on the CH-7α activity in HepG2 cells and to determine the molecular basis, if any, for the observed effect.

Materials and Methods

Culture conditions. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Eagle's Minimum Essential Medium with non-essential amino acids in 150 cm^2 flasks (Corning) maintained at 37° in a humidified atmosphere of 5% CO_2, 95% air. The medium was supplemented with 5% fetal bovine serum (Hyclone), 5% new-born calf serum (Hyclone), penicillin (150 U/mL), streptomycin (150 μg/mL), and amphotericin B (0.38 μg/mL) (Sigma Chemical Co., St. Louis, MO). The medium was changed every third day and the cells were subcultured at a split ratio of 1:3 at confluence (usually 7–8 days). The cells used in this study were 10 to 11-days-old following subculture.

Isolation of the microsomal fraction. The cells were treated with 1 mM diethyl maleate (DEM, Sigma Chemical Co.)-containing serum-free medium for 2 hr. Control flasks received an equivalent volume of serum-free medium only. At the end of the treatment period, the medium was removed and the flask was washed with 10 mL of Dulbecco's phosphate-buffered saline (DPBS). The cells were harvested by scraping with a rubber policeman and washed with 12 mL of DPBS. The cell pellet was transferred to a microcentrifuge tube and disrupted by freezing in liquid N_2 and thawing in the presence of a hypotonic buffer (30 mM potassium phosphate, 1 mM EDTA, pH 7.4). The cells were homogenized in a Dounce homogenizer with 25 strokes of pestle B in 1 mL of the hypotonic buffer. Subsequently, 1 mL of 0.1 mM potassium phosphate buffer, pH 7.4, was added to the homogenate and the cells were further homogenized with 25 strokes of pestle B. The microsomal fraction was obtained as described [13]. The microsomal pellet was resuspended in 0.1 mM potassium phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM NaF, 0.5 mM EDTA, and 25 mM nicotinamide, and used as the enzyme source.

CH-7α assay. CH-7α activity was assayed at 37° in a final volume of 1 mL using the isotope incorporation method of Shefer *et al.* [14]. Microsomal protein concentration was determined by the Hartree [15] modification of the Lowry procedure. CH-7α activity is expressed as dpm/mg microsomal protein/hr.

GSH assay. Following treatment with DEM, the medium was removed and the flask was washed with 10 mL of DPBS. The cells were harvested as described above and resuspended in 700 μL of 0.9% saline. For GSH determination, an aliquot of the cell suspension was added to an equal aliquot of 4% sulfosalicylic acid (SSA). A second aliquot of the suspension was added to 0.4 mL of 1 N NaOH for determination of protein content as described above. GSH concentration of the SSA extract was measured using the GSH-*S*-transferase method of Asaoka and Takahashi [16] and cellular GSH content was expressed as nmol/mg protein.

Probes used in the assay for CH-7α mRNA. A 2172 bp cDNA clone of rat CH-7α in pBluscript SK vector was a gift from Dr. David Russell, University of Texas Southwestern Medical Center. For use as a probe, the insert was excised from the vector with Eco RI and purified by agarose gel electrophoresis. The rat probe is 82% similar to the human gene [17] making it a good cross species probe. As a control, a 1.93 kb cDNA clone of human β chain of fibrinogen in pGEM-3 vector was obtained from Dr. Samar N. Roy, Lindlsey F. Kimball Research Institute. For use as a probe, the insert was excised from the vector with Pst I and purified by agarose gel electrophoresis. All probes were labeled with [^{32}P]dCTP (Amersham) by the random primer method using the Promega Prime-a-Gene system.

Northern blots. Poly A+ enriched RNA was prepared, using the Invitrogen FastTrack mRNA isolation system, from 3×10^7 HepG2 cells that had been treated with 1 mM DEM for 2 hr or control cells. Equal amounts of poly A^+ enriched RNA (15 μg) from treated and control HepG2

* Abbreviations: CH-7α, cholesterol 7α-hydroxylase; GSH, glutathione; DEM, diethyl maleate; DPBS, Dulbecco's phosphate-buffered saline and SSA, sulfosalicylic acid.

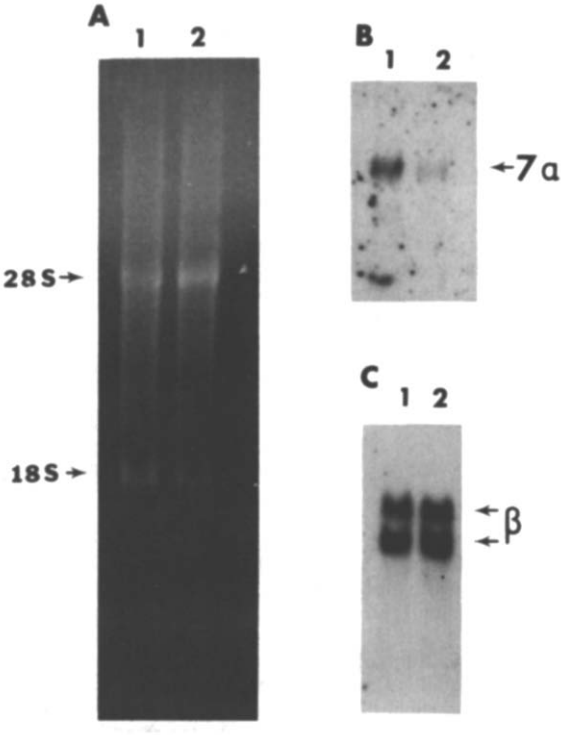

Fig. 1. Northern blot analysis of RNA in control and HepG2 cells treated to remove GSH. (A) Ethidium bromide stained lanes containing 15 μg of poly A^+ enriched RNA from control (lane 1) and treated (lane 2) cell cultures. (B) After transfer to nitrocellulose, the RNA seen in A was hybridized with a cDNA probe for rat 7α-hydroxylase. (C) The blot was stripped off the 7α-hydroxylase probe and rehybridized with a human β fibrinogen cDNA probe.

cells were subjected to electrophoresis on a 1.6% agarose-formaldehyde gel. The RNA was transferred to nitrocellulose by the capillary blotting method. The blots were hybridized with a CH-7α probe in Stratagenes QuikHyb® solution, washed and subjected to autoradiography. Subsequently, the CH-7α probe was stripped off and the same blot was rehybridized with the human β fibrinogen probe. The CH-7α and β fibrinogen Northern blots were exposed for 4 days and 6 hr, respectively.

Results and Discussion

Two hours of treatment with 1 mM DEM depleted HepG2 cell GSH content to 8.8 ± 6.2% (mean ± SEM, N = 3) of the control value of 10.6 ± 1.0 nmol/mg protein (mean ± SEM, N = 3). CH-7α activity in GSH-depleted cells was 47.7 ± 16.0% (mean ± SEM, N = 3, $P < 0.05$) of that in the control (444 ± 57 dpm/mg/hr; mean ± SEM, N = 6; [^{14}C]cholesterol specific activity: 4×10^{12} dpm/mol) cells, a finding in agreement with previous studies [6–8] showing that CH-7α activity was reduced significantly in rat livers depleted of GSH content.

The hybridization of CH-7α cDNA to a Northern blot of poly A^+ enriched RNA from control and treated HepG2 cells is shown in Fig. 1B, lanes 1 and 2, respectively. The rat CH-7α cDNA probe hybridized to one major 3.6 kb band (arrow, 7α). The level of the major 3.6 kb CH-7α mRNA in the control (lane 1) sample is approximately 3- to 4-fold higher than in the treated sample (lane 2). That this reduction in CH-7α mRNA was not due to a general degradation of cellular RNA in the treated samples is supported by the ethidium bromide staining of 28S and 18S rRNA, still present at a discernable level in the poly A^+ enriched preparations (Fig. 1A, lanes 1 and 2). A comparison of the ethidium bromide stained 28S and 18S rRNA in the control (lane 1) and the treated sample (lane 2) shows that the rRNA bands were still intact and that the treated sample lane contained slightly more RNA than did the control sample. Additionally, when this Northern blot was stripped of the CH-7α probe and rehybridized with a probe for human β fibrinogen, the typical pattern of two main mRNA species (1.9 kb and 1.6 kb, arrows, β) for β fibrinogen [18] was detected in both control and treated samples (Fig. 1C, lanes 1 and 2, respectively). In agreement with the levels of rRNA in lane 2 based on ethidium bromide staining, the fibrinogen level in lane 2 was slightly higher than that in the control sample. Specifically, treatment of the HepG2 cells to lower endogenous GSH levels resulted in a *selective decrease* of the CH-7α mRNA over that of β fibrinogen. Equivalent amounts of RNA were recovered from equal numbers of treated and control cells, and ethidium bromide staining indicated that rRNAs were not affected by the treatment.

Taken together, the above findings suggest that the decrease in CH-7α activity in GSH-depleted cells could be due either to a change in mRNA transcription or mRNA stability or to a modification of the enzyme. The results of the Northern blot (Fig. 1B) show that the reduction in steady-state mRNA levels with GSH depletion was commensurate with the decrease in enzyme activity. Thus, while it is not possible to exclude changes in enzyme function under the conditions of the present study, it is likely that the main effector of the loss in enzyme activity resides at the level of the mRNA and is not due to a modification of the enzyme. As with the studies of Jelinek *et al.* [11], it is not possible to ascribe the decrease in CH-7α mRNA levels solely to a decrease in transcription since this could also be accomplished by an increase in the rate of mRNA turnover. While this point remains to be resolved experimentally, two observations support the notion that GSH levels affect CH-7α-mRNA transcription rather than mRNA stability. First, the induced changes in the treated cells was not a general effect, since the *total RNA, rRNA and β fibrinogen mRNA levels were unaffected by the depletion of GSH.* Second, studies on other genes involved in cholesterol metabolism and the CH-7α gene implicate transcriptional regulation [19, 20]. The mechanism whereby GSH levels regulate transcription of CH-7α mRNA remains to be elucidated.

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