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Glutathione and bile acid synthesis. II. Effect of hepatic glutathione content on the activity and mRNA levels of cholesterol 7α-hydroxylase in the rat

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Abstract—Hepatic cholesterol 7α-hydroxylase (CH-7α) activity in intact rats depleted of glutathione (GSH) was reduced significantly ($P < 0.007$) compared with that in untreated controls. Northern blot analysis of poly A^+ mRNA isolated from GSH-depleted and control rat livers showed that there was a reduction in mRNA for CH-7α in treated rats that was commensurate with the reduction in CH-7α activity. The fact that the level of transferrin mRNA was 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 extends previous *in vitro* findings and provides strong justification for a more detailed biochemical investigation into the interaction between GSH levels and the regulation of CH-7α mRNA levels.

In a recent study [1], we showed that the activity of cholesterol 7α-hydroxylase (CH-7α,* EC 1.14.13.17), the rate-limiting enzyme in the catabolism of cholesterol to bile acids [2], is reduced significantly in HepG2 cells (a well-differentiated human hepatoma cell line [3]) depleted of glutathione (GSH). More importantly, we showed that such a treatment is also associated with a reduction in CH-7α-specific mRNA [1]. The nature of the link between cellular GSH content, CH-7α activity and CH-7α mRNA is not known. However, before undertaking a more detailed investigation into the above phenomenon, it was important to demonstrate a similar phenomenon *in vivo*. Thus, the objective of this study was to examine the effect of depleting hepatic GSH content in the intact rat on the activity and mRNA content of CH-7α.

Materials and Methods

Chemicals. [4-^{14}C]Cholesterol was obtained from New England Nuclear, mixed with 99+% pure cholesterol (Sigma Chemical Co., St. Louis, MO), and purified by thin-layer chromatography. Specific activity of the labeled cholesterol used was 4.5×10^{12} dpm/mol. Diethyl maleate (DEM), *d,l*-buthionine sulfoximine (BSO), and GSH-*S*-transferase were obtained from the Sigma Chemical Co. and used as supplied. 7α-Hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol were all obtained from Steraloids (Wilton, NH). All other chemicals and solvents were of reagent grade or better and used as supplied.

Animals and treatment. Male Sprague–Dawley rats weighing between 70 and 80 g were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and maintained under standard conditions in our animal care facility for 7 days before use. Food and water were available *ad lib*. On the day of the experiment, the animals were randomly assigned to either a control group or a treatment group. The animals in the treatment group were injected i.p. with 0.2 mL of a DEM:corn oil (1:1) mixture [4] and 1.0 mL of a solution of BSO (60 mg/mL saline). Control animals received similar volumes of vehicles only. The animals were returned to their cages and allowed free access to food and water. Four hours after the treatment, the animals were killed by decapitation and the livers were removed for the assays described below.

Assay of CH-7α activity. A portion of the liver was rinsed in chilled 1.15% KCl and the microsomal fraction was obtained as described by Guengerich [5]. The microsomes were resuspended in 10 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (w/v) glycerol and used as the enzyme source. CH-7α activity was assayed at 37° in a final volume of 1 mL using the isotope incorporation method of Shefer *et al.* [6]. Microsomal protein concentration was assayed using the Hartree [7] modification of the Lowry procedure. CH-7α activity is expressed as picomoles per milligram of microsomal protein per minute.

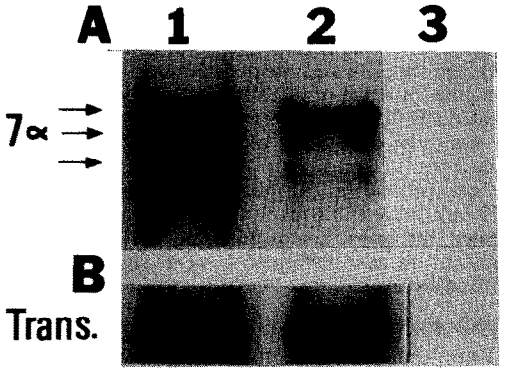

Fig. 1. Northern blot analysis of poly A^+ mRNA extracted from livers of control rats and rats depleted of GSH (two rats/group), and rat prostate. (A) CH-7α hybrids of control (lane 1) and treated (lane 2) rats. Rat prostate RNA was run in lane 3. (B) The blot was stripped of the 7α-hydroxylase probe and rehybridized with a transferrin cDNA probe (lanes 1 and 2).

Assay of hepatic GSH content. A portion of the liver (0.8 to 1.5 g) was accurately weighed and homogenized in 5 mL of chilled 4% sulfosalicylic acid (SSA). GSH concentration of the SSA extract was assayed using the GSH-*S*-transferase method of Asaoka and Takahashi [8] and GSH content was expressed as micromoles per gram.

Probes used in the assay for CH-7α mRNA. A 2172 bp cDNA clone of rat CH-7α in pBluescript 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. A 688 bp cDNA clone of rat transferrin in pSP65Tf vector was a gift from Dr. Michael Griswold, Washington State University, and used as an internal control. For use as a probe, the insert was excised from the vector with *Eco*RI and *Pst*I and purified by agarose gel electrophoresis. All probes were labeled

* Abbreviations: CH-7α, cholesterol 7α-hydroxylase; GSH, glutathione; BSO, buthionine sulfoximine; DEM, diethyl maleate; and SSA, sulfosalicylic acid.

with [^{32}P]dCTP (Amersham) by the random primer method using the Promega Prime-a-Gene system.

Northern blots. Liver total RNA was isolated from control and treated rats (two rats/group; RNA pooled) using the guanidinium thiocyanate–phenol–chloroform method [9]. Purified total RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water. The purity and concentration of the RNA were determined by ultraviolet absorbance (260/280) in a Beckman DU40 spectrophotometer. Poly A$^+$ RNA was prepared using the Promega PolyATtract System 1000 mRNA isolation system from the previously isolated total liver RNA. Equal amounts of poly A$^+$ RNA (8 μg) from control and treated livers were subjected to electrophoresis on a 1.6% agarose-formaldehyde gel. Rat prostate poly A$^+$ RNA was run beside the liver poly A$^+$ RNAs as a control tissue. The RNA was transferred to Immobilon N (Millipore Corp.) by the capillary blotting method. The blots were hybridized with the CH-7α probe in Stratagene's QuikHyb^{TM} solution, washed and subjected to autoradiography. Subsequently, the CH-7α probe was stripped off and the same blot was rehybridized with the rat transferrin probe. The CH-7α and rat transferrin Northern blots were exposed to X-ray film for 24 and 1 hr, respectively. The autoradiograms were quantified by densitometry using an LKB scanning laser densitometer (Pharmacia, LKB Biotechnology, Piscataway, NJ), and the peak areas were determined using the GSXL computer program from LKB. Peak areas for control and treated CH-7α hybrids were normalized for RNA loading by using the peak areas of the hybridization signal for transferrin.

Statistical analyses. All data are shown as means ± SEM. CH-7α activity and hepatic GSH content in control and treated rats were compared using Student's *t*-test for unpaired means. Differences were considered to be statistically significant at $P < 0.05$.

Results and Discussion

Four hours after treatment with DEM/BSO, hepatic GSH content was depleted to nearly 23% of the control value [control (N = 5): 2.2 ± 0.09 μmol/g vs treated (N = 5: 0.51 ± 0.23 μmol/g, $P < 0.001$]. Hepatic CH-7α activity in the treated animals was reduced by nearly 38% relative to control values (control (N = 5): 7.9 ± 0.7 pmol/mg microsomal protein/min vs treated (N = 5): 4.9 ± 0.5 pmol/mg microsomal protein/min, $P < 0.007$), a finding in agreement with previous studies [4, 10, 11] 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$^+$ RNA from control and treated rat livers is shown in Fig. 1, lanes 1 and 2, respectively, and rat prostate poly A$^+$ RNA, lane 3. The rat CH-7α cDNA probe hybridized to three major bands as expected for rat liver (arrows) [12] and not at all to rat prostate poly A$^+$ mRNA. The level of the CH-7α mRNAs in the treated (lane 2) sample was reduced to 33% of that in the control samples (lane 1). That this reduction in CH-7α mRNA was not due to a general degradation of cellular RNA in the treated samples was supported by hybridization of the same blot to rat transferrin, Fig. 1B, lanes 1 and 2. Densitometry of the transferrin hybridization indicated that equal levels of transferrin were present in both control and treated samples. Specifically, treatment of rats to lower endogenous GSH levels resulted in a *selective decrease* of the CH-7α mRNA over that of transferrin.

Taken together, the above *in vivo* findings are in general agreement with previous *in vitro* [1] findings. Thus, hepatocyte GSH content is linked to CH-7α activity and mRNA. While the nature of the relationship between GSH content and CH-7α activity and mRNA remains to be elucidated, the results of the present study provide sufficient justification for investigating that relationship in detail.

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Department of Veterinary Biosciences
University of Illinois
Urbana, IL 61801, U.S.A.

ASLAM S. HASSAN*
DAVID BUNICK
SUZANNE H. ST. DENIS
LISA A. LUND

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* Corresponding author: Dr. Aslam S. Hassan, Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign, 3516 Veterinary Medicine Basic Science Bldg., 2001 South Lincoln Ave., Urbana, IL 61801. Tel. (217) 333-7851; FAX (217) 333-4628.