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SHORT COMMUNICATION

Effect of Ethanol on the Expression of Hepatic Glutathione *S*-Transferase: An *In Vivo/In Vitro* Study

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ABSTRACT. Ethanol, a human toxicant and a solvent in pharmacological research, is known to interfere with biotransformation of xenobiotics. We compared the *in vivo* and *in vitro* long-term effects of ethanol exposure on the expression of glutathione *S*-transferases (GST, EC 2.5.1.18) in rat liver. Long-term *in vivo* ethanol treatment to achieve blood ethanol levels ranging between 10–50 mM was by liquid diet feeding. For *in vitro* experiments, rat hepatocytes co-cultured with rat liver epithelial cells were exposed to 17 and 68 mM ethanol for up to 10 days. Two weeks of liquid diet ethanol treatment increased total GST activity. Both Mu and Alpha classes and in particular the A1 and A2 subunits and the amount of their corresponding mRNAs were increased. Total GST activity was also increased in co-cultures after exposure to 68 mM ethanol for 10 days. However, the Mu class subunits M1 and M2 and the corresponding mRNAs were increased, rather than the Alpha class subunits. Thus, long-term exposure to ethanol induces hepatic GST both *in vivo* and *in vitro*, but different isoenzymes are affected. Consequently, extrapolation of *in vitro* data on GST expression and regulation to the *in vivo* situation must be judicious. During xenobiotic metabolism in cell culture, a shift in relative expression and induction of different GST forms may occur, resulting in either an under- or overestimation of effects. BIOCHEM PHARMACOL **60**;10:1491–1496, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. rat liver; rat hepatocyte co-culture; ethanol; glutathione *S*-transferase; induction; *in vitro–in vivo* extrapolation

Ethanol is often used in pharmaco-toxicological research and testing as a solvent for water-insoluble substances. However, in man as well as in animals, ethanol exposure has been associated with both induction and inhibition effects on the hepatic phase II glutathione *S*-transferase (GST, EC 2.5.1.18)** system [1–4]. Consequently, the balance between toxification and detoxification of xenobiotics metabolized through the GST pathway could be drastically affected by ethanol exposure. Differences in methodology and, in particular, the length and amount of ethanol exposure are at the origin of highly variable data with respect to GST expression in rat liver [1–6]. Therefore in this study, the effect of a continuous ethanol exposure for 2 weeks on the expression of GST was investigated both in rat liver and in co-cultures of rat hepatocytes with rat liver epithelial cells.

The GST protein family comprises several isoenzymes that have been divided into seven classes [7–10]. By catalyzing nucleophilic attacks of S-atoms of glutathione molecules on the electrophilic center of substrates, protein–glutathione conjugates are formed. GSTs are localized in the cytosol as homo- or heterodimeric proteins [11]. The major GST subunits expressed in the adult liver are Alpha class subunits A1, A2, and A3 and the Mu class subunits M1 and M2. GST subunit P1, a member of the Pi class, is only expressed in fetal hepatocytes, cultured rat hepatocytes, and during early stages of hepatocarcinogenesis [7, 12].

Co-culture of rat hepatocytes with primitive biliary rat epithelial cells has been chosen as *in vitro* model, since the activities of most phase I and II biotransformation enzymes remain expressed for at least one week of culture [13–16]. Major phase I biotransformation pathways, including cytochrome P450-dependent mono-oxygenation [16, 17] and the phase II GST detoxification system [18], also remain

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** *Abbreviations:* EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GST, glutathione *S*-transferase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; and RT-PCR, reverse transcriptase–polymerase chain reaction.

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inducible. The latter pathway has been studied in co-cultured rat hepatocytes at the enzyme, protein, mRNA, and transcriptional levels [12, 16, 19, 20].

MATERIALS AND METHODS

Chemicals

Crude collagenase type I, BSA (fraction V), bovine insulin, CDNB, glutathione, EA, EDTA, and epoxy-activated Sepharose 6B were obtained from Sigma Chemical Co. All culture media, fetal bovine serum, and trypsin–EDTA solution were purchased from GIBCO. DCNB and absolute alcohol came from Merck-Schuchardt. NBD-Cl was obtained from Janssen Chimica. RNazol B was purchased from A.M.S. Biotechnology. Klenow fragment of DNA polymerase was from Pharmacia Biotech Ltd. All other chemicals were of reagent grade and were purchased from general commercial sources.

Animals

Male Sprague–Dawley rats, initially weighing 220 to 240 g, were maintained on a standard R3 diet (Ewos AB). However, during administration of ethanol to rats, control animals were pair-fed for 2 weeks a commercial nutritionally adequate diet containing 18% and 35% as protein and fat, respectively (expressed in Joules). The remaining 47% was supplied as carbohydrates. In the ethanol-containing diet, ethanol made up 36% with the remaining 11% from carbohydrates. This low carbohydrate diet had no significant effect on the levels of GST [21]. During the 2-week treatment period, a steady increase in body weight in both controls (230 to 329 g) and ethanol-treated animals (231 to 307 g) was observed.

Isolation and Preparation of Hepatocyte Co-cultures

Hepatocytes were isolated [22] from untreated male Sprague–Dawley rats (200–250 g) and co-cultured with rat liver epithelial cells obtained from 10-day-old Sprague–Dawley rats as described in detail by Vanhaecke *et al.* [23]. Co-cultured hepatocytes remained either untreated or were continuously exposed to final concentrations of either 17 or 68 mM ethanol for 10 days. The culture media were renewed every day.

Enzyme Assays

Enzyme activities were measured in cytosolic fractions prepared from rat liver, freshly isolated (T0) and co-cultured hepatocytes either untreated or treated with ethanol for 10 days. Total, Mu, Alpha, and Pi class GST activities were measured towards the substrates CDNB [24], DCNB [24], NBD-Cl [25], and EA [26], respectively. The cytosolic protein content was measured using a Bio-Rad Protein Assay kit (Bio-Rad) based on the principle of Bradford [27] with BSA as a standard. The reproducibility of the various GST enzyme assays was characterized by a coefficient of variation ranging between 4.4 and 9.7%.

GST proteins

Individual GST subunits and total GST content were quantified in cytosolic fractions using glutathione-affinity chromatography [12] followed by reversed-phase HPLC [28].

Northern Blot Analysis

Total RNA was isolated from frozen cell pellets using RNazol B and the procedure described by Chomoczynski and Sacchi [29]. Equal amounts of total RNA (15 μg per lane) were separated by electrophoresis in a denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham) [30]. Filters were prehybridized and hybridized with full-length [α-^{32}P]dCTP-labeled GST cDNA probes according to Feinberg and Vogelstein [31]. After hybridization, filters were washed, dried, and autoradiographed at −80° using Fuji RX-NIF x-ray film. Hybridization signals were quantified using a Bio-Rad image analysis densitometer. The signals were corrected for any unequal loading of the cellular RNA by normalization to the 28S rRNA content. The cDNA probes complementary to GST A1 and A2 mRNA (cross-hybridizes to GST A3 mRNA), GST M1 mRNA (cross-hybridizes to GST M2 mRNA), and GST P1 were a kind gift from Dr. S. Pemble (University College London, U.K.) [32].

RT–PCR Analysis

Due to sample contamination, only the levels of GST A1 and A2 mRNA in rat liver cytosol could be estimated. GST A1 and A2 sequences were amplified from total rat liver RNA according to Lindros *et al.* [33], except that here 100 pmol (GST A1) and 50 pmol (GST A2) of each primer were used in the PCR reactions. The amount of GST A1 and A2 transcripts in each sample was normalized to the relative amplification of the same cDNA with β-actin primers, as described previously [34]. The relative amounts of the PCR products were quantified by anion exchange HPLC [35] and the data obtained were analyzed as proposed by Lindros *et al.* [33].

RESULTS AND DISCUSSION

In agreement with Hétu *et al.* [3] and Munoz *et al.* [4], we found an increase in total GST activity in rat liver after long-term ethanol treatment (Fig. 1A). Ethanol significantly induced both Alpha and Mu class activities by 36% and 44%, respectively. As ethanol has also been shown to induce hepatic lipid peroxidation [36, 37], it is suggested that the production of organic peroxides, which are typical Alpha class substrates [38], is increased. Therefore, the induction of Alpha class GST activity might be an adaptive

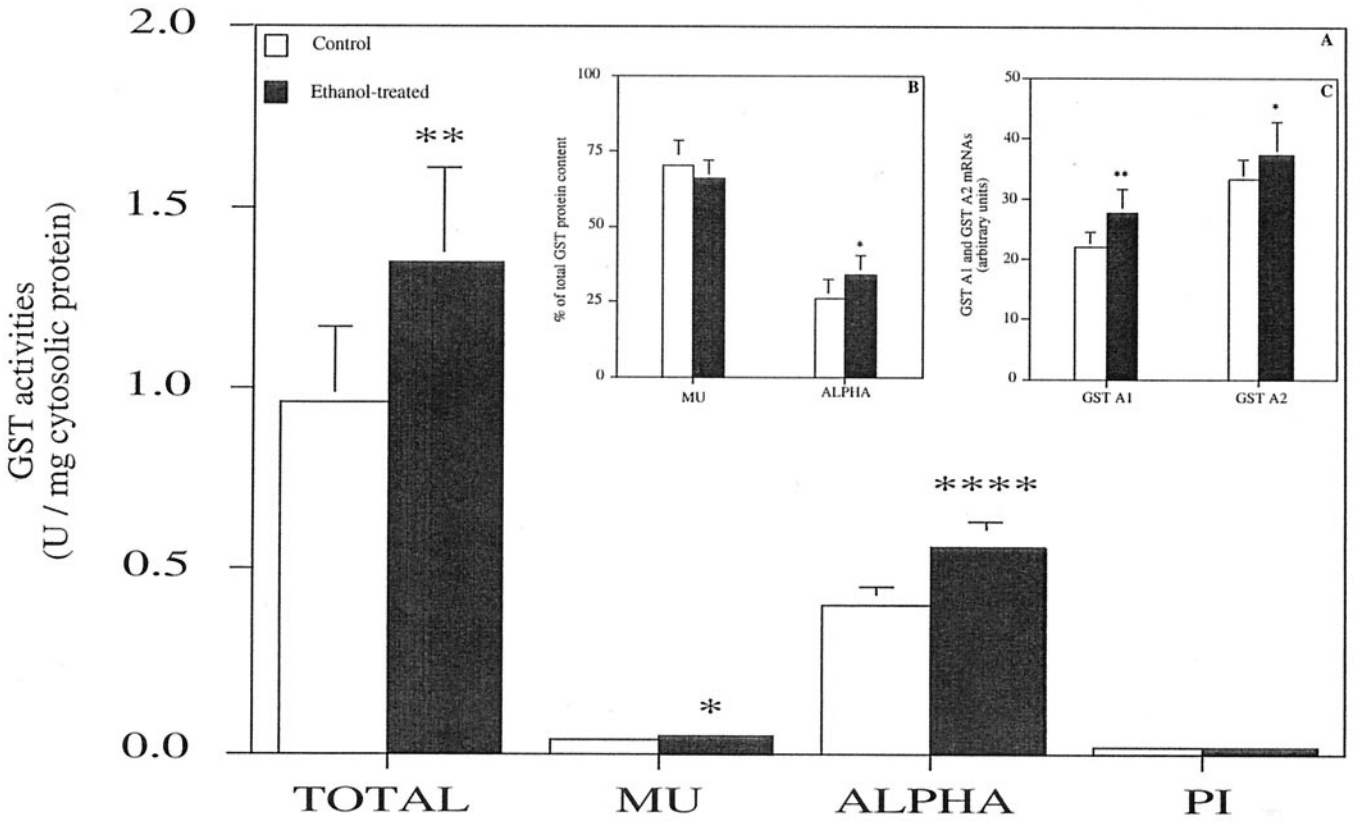

FIG. 1. Induction of male rat hepatic GST isoenzyme activities (A) and proteins (B) and GST A1 and A2 mRNAs (C) upon a 2-week exposure to ethanol by liquid diet feeding ($n = 10$ for untreated and ethanol-treated group). Each value represents the mean ± SD. Results obtained at the protein level (B) are expressed as a percentage of the total GST protein content that was set arbitrarily to 100%. The mean total GST protein contents obtained for control and ethanol-treated rats were 20.79 ± 4.68 and 28.58 ± 7.64 μg/mg protein, respectively. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$ for significant difference between control and ethanol-treated animals according to an unpaired Student's *t*-test.

response to ethanol-induced lipid peroxidation [36]. No effect of ethanol could be detected on the Pi class GST activity, which showed levels of almost zero, in line with evidence of its absence in adult rat liver [39]. *In vivo* data on individual GST proteins [5] and corresponding mRNAs are rather scarce. Here, we report a significant increase in the total amount of GST proteins in rat liver after ethanol treatment, which is due to increased amounts of Alpha class subunits (Fig. 1B), in particular A1 and A2. Moreover, the increase in GST A1 and A2 proteins in response to ethanol is also reflected in an increase in GST A1 and GST A2 mRNAs (Fig. 1C), suggesting that ethanol is acting at the pretranslational level to effect changes in GST A1 and GST A2.

When co-cultures were exposed to ethanol for 10 days, GST activity tended to increase in a dose-dependent way. Total GST activity significantly increased by 35% in cells treated with 68 mM ethanol. The isoforms responsible for this increase in GST activity could not be identified, since no statistically significant effects on Alpha, Mu, or Pi class activities were detected. However, the highest numerical increase (25%) in activity was found for the Mu class isoforms, confirming a previous *in vitro* study done by our group [40]. In contrast to the activity results, there was a statistically significant increase in total GST protein content after a 10-day exposure to 68 mM ethanol, due to an increase in Mu class GST protein content. When compared with the values obtained in control co-cultures, Mu class subunits M1 and M2 were 3- and 2.5-fold increased, respectively. Consequently, their percentage contribution to the total amount of GST proteins was significantly increased in response to exposure to 68 mM ethanol (Fig. 2A). On the contrary, the concentration of Alpha and Pi class isoforms was not significantly altered upon *in vitro* ethanol exposure. All the *in vitro* results obtained at the protein level via HPLC analysis were confirmed by Western blots (not shown). Quantification of the transcript steady-state levels (Fig. 2B), obtained in 3 different hepatocyte co-cultures exposed to 17 and 68 mM ethanol for 10 days, showed that the GST M1/M2 mRNA contents were concentration dependently increased by 102% ($P < 0.05$), and 279% ($P < 0.05$), respectively. With respect to the helper cells, untreated epithelial cell cultures contained 0.7 ± 0.2, 2.4 ± 0.4, and 5.5 ± 0.1 μg/mg cytosolic protein of GSTM1, M2, and P1 subunits, respectively. However, in co-cultures, only 5% of the total GST enzyme activity originated from the epithelial cells, whereas they accounted for about 40% of the total protein content. When pure

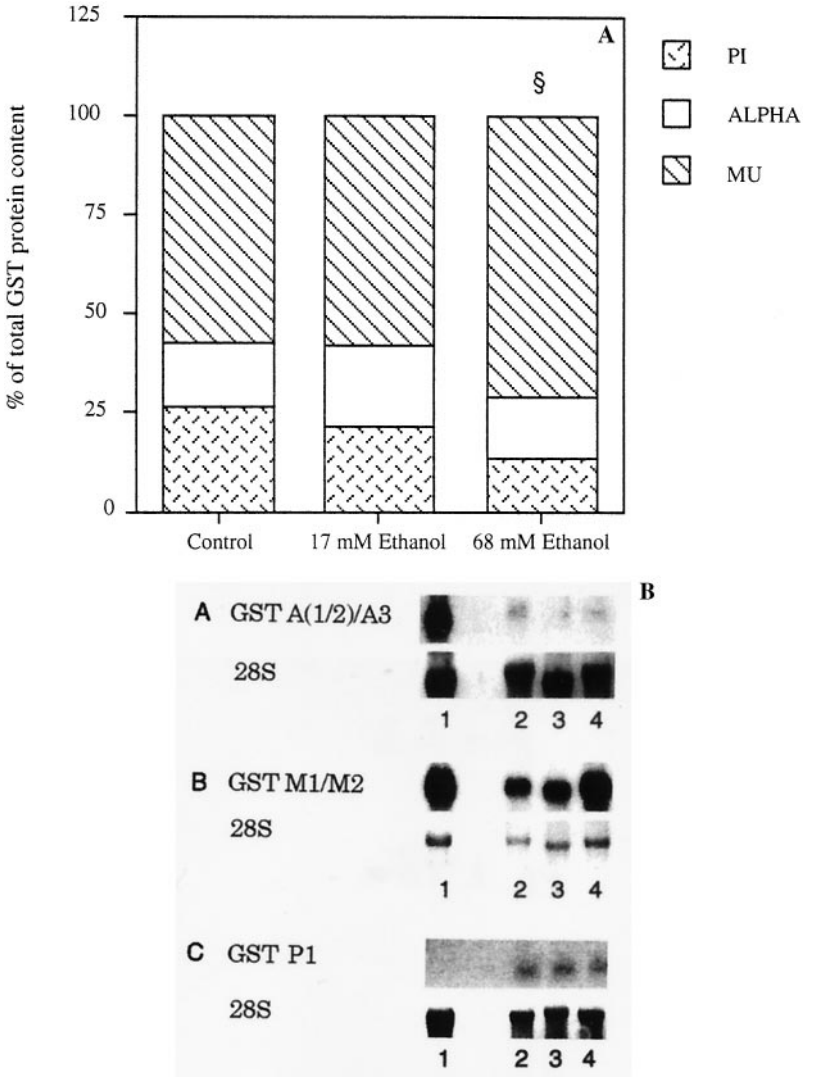

FIG. 2. Effect of a continuous ethanol treatment (17 and 68 mM) for 10 days on GST proteins (A) and mRNAs (B) in co-cultures of adult male rat hepatocytes with primitive biliary epithelial cells. Results are means ± SD of three independent culture experiments. (A) Results are expressed as a percentage of the total GST protein content that was set arbitrarily to 100%. The mean total GST protein contents obtained in cultures either untreated or treated with 17 and 68 mM ethanol were 59.1 ± 18.6, 98.6 ± 39.4, and 125.4 ± 40.8 μg/mg protein, respectively. (B) Northern blot analysis of mRNAs encoding GST subunits A1/A2 (A), M1/M2 (B), and P1 (C) of freshly isolated hepatocytes; and ethanol-treated co-cultures of rat hepatocytes for 10 days. Each lane was loaded with 15 μg of total RNA. The signals were corrected for unequal loading by normalization to 28S rRNA content. Lane 1: freshly isolated hepatocytes; lane 2: control co-culture; lane 3: co-culture with 17 mM ethanol; lane 4: co-culture with 68 mM ethanol. §Significantly different from the control value according to a two-way analysis of variance ($P < 0.05$).

epithelial cell cultures were treated with ethanol, no significant effect was observed on the epithelial cell protein content, but their total GST activity increased by 3.5 and 10% upon exposure to 17 and 68 mM ethanol, respectively. However, the helper cells still represented only about 7% of the activity measured in co-cultures and therefore have not been taken in account. Consequently, even an underestimation of the effects observed in co-cultures is made.

In conclusion, this paper clearly shows that the *in vitro* approach, and more specifically the co-culture system, can be a useful tool for studying the modulatory effects of GST enzyme induction [41]. However, a discrepancy exists between the individual GST isoenzymes affected by ethanol *in vitro* (Mu) and *in vivo* (Alpha), and thus it is crucial to interpret the *in vitro* data with great care and take into account the limits of the culture system employed. It should, however, be emphasized that previous reports clearly showed that the co-culture system used is able to handle both basic and exogenously induced oxidative stress [42, 43]. The latter phenomenon is expected to occur because of exposure to ethanol [36, 37, 44]. Therefore, the discrepancy in induction of Mu class isoforms we observe in co-cultures may represent a direct effect of ethanol under more 'stress-free' conditions. In this respect, it would be interesting to investigate the effects of ethanol on GST expression in pure hepatocyte cultures that suffer more from oxidative stress than co-cultures do [43].

The results of our study also clearly illustrate that in experiments in which inducing effects of the solvent need to be avoided, ethanol should not be used. It can be replaced by an alternative delivery system, e.g. cyclodextrin, which has neither inducing nor inhibiting effects on the drug metabolism pathway examined.*

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