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Regulation of glutathione S-transferase subunits 3 and 4 in cultured rat hepatocytes

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mRNA levels of glutathione S-transferase (GST) subunits 3 and 4 were measured with a specific cDNA probe in adult rat hepatocytes maintained either in conventional culture or in coculture with rat liver epithelial cells. Four media conditions were used, i.e. with or without fetal calf serum (FCS) and with nicotinamide or dimethylsulfoxide (DMSO). When FCS was present in the culture medium, GST subunit 3 and 4 mRNAs were expressed at a level close to that found in freshly isolated hepatocytes during the whole culture period both in conventional culture and in coculture. All other culture conditions resulted in an increase of GST 3 and 4 mRNA levels. After exposure to phenobarbital an increase in GST 3 and 4 mRNA levels was demonstrated in both culture systems. Comparison with previous findings on the expression of GST subunits 1, 2 and 7 in the same culture conditions indicates that the different classes of GST are regulated independently.

Glutathione S-transferase; Rat hepatocyte; Phenobarbital; (In vitro)

1. INTRODUCTION

Glutathione S-transferases (GST, EC 2.5.1.18) catalyze nucleophilic attack of glutathione on electrophilic centers in a wide variety of organic molecules [1,2]. The latter include xenobiotics as well as compounds endogenous to the organism. Three classes of cytosolic GST (α, μ and π), common to several mammalian species, have been identified [3]. Rat GST subunits 1 and 2 belong to class α, while GST formed by combination of subunits 3, 4 and 6 are members of class μ [3]. Subunit 7 has been assigned to class π [4]. The different subunits can be differentiated by a combination of two physical parameters, namely apparent subunit molecular weight and isoelectric point [5] and substrate specificity is an additional distinguishing feature [6].

Each rat tissue has a characteristic distribution of GST isozymes which can change during development and may be modulated by endogenous compounds such as hormones, or exogenous compounds including carcinogens and inducers of drug-metabolizing enzymes [7,8]. The mature liver contained the highest amount of GST and in order to elucidate mechanisms implicated in the regulation of GST, hepatocyte cultures are considered as a valuable experimental model. Recent studies showed that expression of GST mRNAs, profile of GST isozymes and GST activity levels varied depending on the culture conditions, i.e. the composition of the medium and whether hepatocytes were in conventional culture or coculture [9–11].

In order to study expression of the GST μ family in conventionally cultured and cocultured hepatocytes, a specific cDNA probe (Pemble and Taylor, unpublished results), was used to determine steady state mRNA levels for subunits 3 and 4 in rat hepatocytes cultured in 4 different media conditions, i.e. media with or without fetal calf serum (FCS) and media with nicotinamide or dimethylsulfoxide. These media have been previously found to differently affect subunits 1, 2 and 7 expression [10,11]. Moreover, the effect of phenobarbital which is known to induce GST subunits 3 and 4 in vivo [13,14], was also investigated.

2. MATERIALS AND METHODS

2.1. *Materials*

Culture media and FCS were obtained from Gibco, Scotland. Collagenase, bovine albumin, bovine insulin were products from Sigma Chemical Co., USA. The Nick translation kit and [^{32}P]dCTP were obtained from Amersham, England. All other compounds were readily available commercial products.

2.2. *Cell isolation and culture*

Hepatocytes from adult male Sprague Dawley rats weighing 180–200 g were isolated by perfusion of the liver with a collagenase solution as previously described [15]. They were seeded at a density of 10×10^6 cells per 175 cm^2 flask in 25 ml of medium containing

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10% FCS. The standard medium (St) consisted of 75% minimal essential medium and 25% medium 199, containing 0.2 mg/ml bovine serum albumin, 10 μg/ml bovine insulin and 10% FCS. The medium was first changed 4 h after cell seeding and every day thereafter. Cocultures were set up by adding 14×10^6 rat liver epithelial cells (RLECs) per flask in order to get cell confluency within 24 h [16]. Four hours after hepatocyte seeding 7×10^{-5} M and 3.5×10^{-6} M hydrocortisone hemisuccinate was added to conventional cultures and cocultures respectively. These concentrations remain unchanged during the culture period. Four different media which are representative of changes in GST subunit profiles and activity were tested, namely St medium minus FCS (St – FCS), St medium with FCS (St + FCS), St – FCS containing 25 mM nicotinamide (St – FCS + Nic) and a mixture of St medium (50%)/Williams' E medium (50%) minus FCS with 2% dimethylsulfoxide (St/W – FCS + DMSO).

Sodium phenobarbital at the final concentration of 3.2 mM was added to conventional cultures 4 h after cell seeding and to cocultures when cell confluency was reached, i.e. about 24 h later. Thereafter this barbiturate was added every day at the time of medium renewal.

2.3. *Northern blot and hybridization with cDNA probes*

Total RNA was extracted from cultured hepatocytes by the guanidium-thiocyanate-cesium chloride method [17,18]. RNA (10 μg) was subjected to electrophoresis in a denaturing formaldehyde-agarose gel and transferred onto Hybond-N sheets. These sheets were prehybridized and hybridized with ^{32}P-labelled probes. 'JT 9L' is a rat GST subunit 4 cDNA sequence cloned with *Eco*RI linkers into pUC 18. The two GST subunits 3 and 4 were detected but not distinguishable with ^{32}P-labelled 'JT 9L' [12]. Albumin mRNA was detected with a ^{32}P-labelled albumin cDNA [19]. After hybridization, sheets were washed, dried and autoradiographed at −80°C. Relative amounts of mRNA were determined by scanning X-ray films with a densitometer.

3. RESULTS

3.1. *GST 3 and 4 mRNA expression in conventionally cultured hepatocytes*

Male rat hepatocytes were maintained in primary culture for 6 days with the 4 different media. Hepatocytes were harvested at days 2, 4 and 6 after plating. After RNA isolation a cDNA probe for GST subunits 3 and 4 was used to measure levels of GST 3 and 4 transcripts. Northern blots (fig.1A) reflect changes in steady state levels of subunits 3 and 4 mRNAs with time in culture. These mRNAs were abundant in freshly isolated hepatocytes. When the cells were cultured in a medium containing FCS, high levels of GST 3 and 4 transcripts were maintained during the whole culture period. In the absence of FCS, subunits 3 and 4 mRNAs content remained unchanged until day 4 of culture and started to increase on day 6. In contrast, when hepatocytes were maintained in a medium with or without FCS, the albumin mRNA level decreased during the 6 days in culture (not shown). Hepatocytes cultured in a medium containing DMSO expressed subunits 3 and 4 mRNAs at a level 1.7–2 times higher than in freshly isolated hepatocytes whatever the culture time. In contrast, when nicotinamide was added to the medium, amounts of these mRNAs decreased after the cells were plated until day 4 and then increased. On day 6 of culture a 2.5-fold elevation of GST 3 and 4 mRNAs was observed. In the presence of nicotinamide or DMSO the albumin mRNA level remained stable over the culture period (not shown).

3.2. *GST 3 and 4 mRNAs expression in cocultured hepatocytes*

Hepatocytes maintained in coculture with RLECs were harvested at days 4, 8, 12 and 16 (fig.1B). Only the first 3 time points were analysed in coculture maintain-

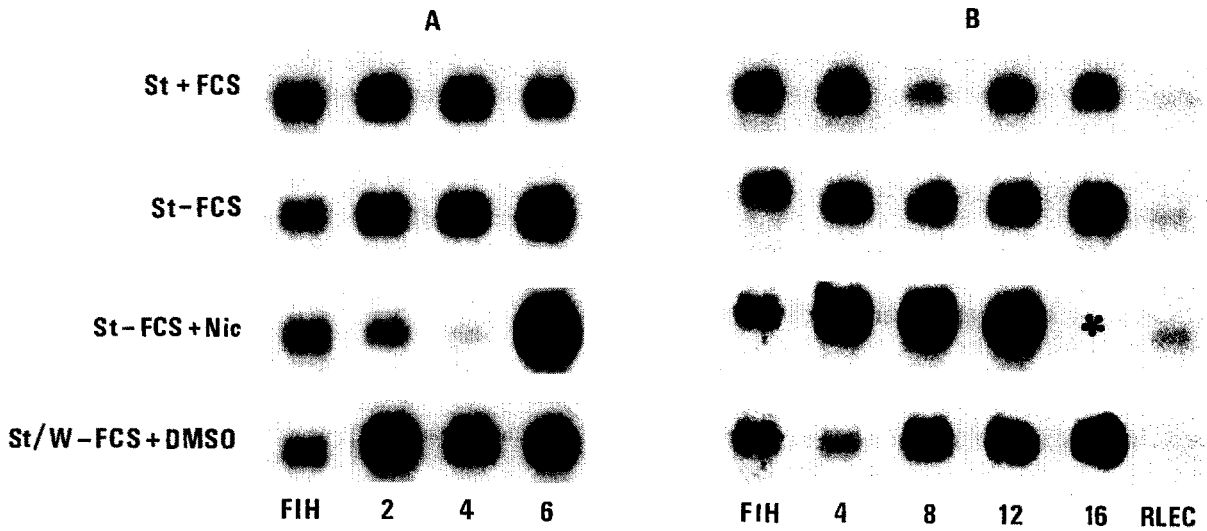

Fig.1. Expression of GST 3 and 4 mRNAs in conventionally (A) and cocultured (B) hepatocytes. In conventional culture RNA was extracted from hepatocytes cultured for 2, 4 and 6 days with 4 different media: St – FCS; St + FCS; St – FCS + Nic; St/W – FCS + DMSO. In coculture RNA was isolated after 4, 8, 12 and 16 days of culture with the same media. Only one band of approximately 1100 nucleotides in length was detected (FIH, freshly isolated hepatocytes; *, not determined).

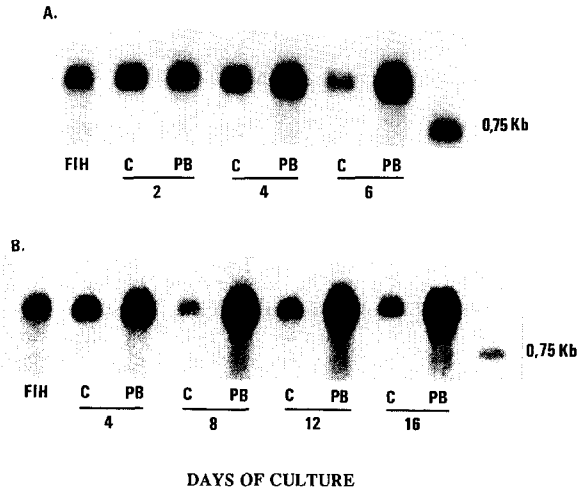

Fig.2. Effect of phenobarbital on the GST 3 and 4 mRNAs expression in conventionally cultured (A) and cocultured (B) hepatocytes. In conventional culture RNA was isolated from control (C) and phenobarbital-treated hepatocytes (PB) after 2, 4, and 6 days. In coculture, RNA was extracted from control (C) and PB-treated cells (PB) after 4, 8, 12, and 16 days of culture.

ed in St − FCS + Nic. When hepatocytes and RLECs were cocultured in a medium containing FCS, subunits 3 and 4 mRNAs levels remained unchanged compared with those measured in freshly isolated hepatocytes over the culture period. In the absence of FCS, the levels of transcripts increased after cell seeding and a 2-fold elevation was observed after 16 days. When nicotinamide was added to the medium, GST 3 and 4 mRNA content increased by 2-fold from day 4 to day 12 of culture whereas in the presence of DMSO a slight decrease was observed on day 4, then the mRNA levels increased for up to day 16. RLECs contained only few amounts of GST 3 and 4 mRNAs and consequently barely contributed to the total amount of GST 3 and 4 mRNAs found in coculture (fig.1B). The amount of albumin mRNA was close to that found in freshly isolated hepatocytes and remained stable over the culture period (not shown).

3.3. *Effect of phenobarbital on GST 3 and 4 mRNA levels*

In conventional culture, phenobarbital increased GST 3 and 4 transcripts content by 1.4- to 2-fold after 4 and 6 days of treatment respectively (fig.2A). When the barbiturate was added to the medium of coculture, the amount of GST 3 and 4 mRNAs was increased until day 8 and remained relatively stable with a 2.7- to 3-fold elevation compared with non-treated cells (fig.2B).

4. DISCUSSION

Previous studies have shown that profound variations in both GST activity and GST isozymic profile can occur in rat hepatocytes depending on culture conditions [9,10]. In most conditions, the steady-state mRNAs for GST subunits 1 and 2 were decreased while those for subunit 7, found in fetal hepatocytes but absent in adult liver, became expressed in culture [11]. Except when the medium was supplemented with FCS, steady-state mRNAs for GST subunits 3 and 4 increased during culture, illustrating further that the different classes of GST are regulated independently and that the μ class represents an increasing part of total GST levels in vitro. Recently Abramovitz et al. [20] came to the same conclusion from experiments on rat hepatocytes cultured in a serum-supplemented medium for 3 days.

This increase in steady-state mRNAs for GST subunits 3 and 4 was not related to the dedifferentiation process which affects various specific liver functions in various culture conditions [21]. Indeed, except for in cells cultured in an FCS-supplemented medium, GST 3 and 4 mRNA levels were increased without any correlation with changes in albumin mRNA content. In a medium added with FCS, which is known to favor hepatocyte dedifferentiation as shown by the decline in albumin mRNA level, GST subunits 3 and 4 mRNAs remained relatively stable.

The probe used in this study did not allow to separate subunits 3 and 4 mRNAs. However, from previous studies [9,10] it could be supposed that GST 3 and 4 mRNA increase corresponded mainly to an increase in GST subunit 3 mRNA. Indeed by reverse phase HPLC analysis it has been shown that GST 3 subunit was increased while GST 4 subunit remained relatively unchanged in hepatocytes cultured with FCS or nicotinamide [10]. Moreover, GST activity toward 1,2-dichloro-4-nitrobenzene (DCNB) which is a substrate specific for GST forms 3-3 and 3-4 [22], was increased in the presence of DMSO [9].

Administration of phenobarbital to rats enhances in vivo synthesis of subunits 1 and 3 when compared with subunits 2 and 4 respectively [13,14]. When 3.2 mM phenobarbital was added to the culture medium, an increase in GST 3 and 4 mRNAs levels was obtained when compared with the content of non-treated cells either in conventional culture or in coculture. A 3-fold elevation of subunits 3 and 4 transcripts was observed after 8 days in coculture followed by stabilisation of their levels until day 16, whereas in conventional culture only a 2-fold increase was demonstrated after 6 days. This difference in the response to the barbiturate with the two culture conditions could be explained by the better maintenance of the transcriptional activity in cocultured hepatocytes [23]. Variation in DCNB activity which is subunit 3-specific and increase of the amount of GST subunit 3 were observed in cultured rat hepatocytes treated by phenobarbital [24] and seem to reflect changes in steady state of GST 3 and 4 mRNA levels found in this study. Pickett et al. [25] have demonstrated that a transcriptional activation of

subunits 3 and 4 genes occurred after administration of phenobarbital to rat and was sufficient to account for the elevation of GST 3 and 4 mRNAs. Further studies are needed to verify if the GST 3 and 4 transcripts increase in cultured hepatocytes treated with phenobarbital was caused only by an elevation of the transcription rate, as in vivo, or also at least partly by a stabilisation of mRNAs.

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