Pergamon

Biochemical Pharmacology, Vol. 48, No. 1, pp. 139–144, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0092-Y

THE DISTRIBUTION OF NON-SPECIFIC CARBOXYLESTERASES AND GLUTATHIONE *S*-TRANSFERASES IN DIFFERENT RAT LIVER CELLS

EFFECTS OF VITAMIN A DEFICIENCY

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(*Received* 30 *October* 1993; *accepted* 16 *February* 1994)

Abstract—Non-specific carboxylesterases (carboxylesterases) and glutathione *S*-transferases (GSTs) are two groups of drug metabolizing enzymes responsible for hydrolysis and glutathione conjugation of xenobiotics. This study was conducted to determine the following: (1) the distribution of carboxylesterase and GST activities in different rat liver cells, (2) the effects of vitamin A deficiency (A−) on the absolute activities and on the distribution of carboxylesterases and GSTs in rat liver. Rat livers were fractionated into parenchymal and non-parenchymal cells by means of collagenase perfusion and differential centrifugation. Non-parenchymal cells were further fractionated by means of Percoll density gradient centrifugation into a layer of Kupffer cells and another layer containing stellate and endothelial cells. Carboxylesterase and GST activities were determined in these fractions. The results of the study show that: (1) both carboxylesterases and GSTs were mainly localized in the parenchymal fraction, (2) there was no significant difference between male and female rats with regard total activity or distribution of carboxylesterases and GSTs in rat liver cells, (3) A− caused a highly significant reduction in carboxylesterase and GST activities in total liver homogenates and parenchymal cells. This reduction was not ameliorated by administration of retinoic acid 18 hr before sacrifice of animals. These results open up a new era of investigations about the potential role of vitamin A in the regulation of detoxification enzymes.

Key words: vitamin A; non-specific carboxylesterases; glutathione *S*-transferases; liver cells; non-parenchymal cells

Non-specific carboxylesterases (EC 3.1.1.1) and GSTs† (EC 2.5.1.18) are two key enzymes that play important roles in phase I and phase II detoxification reactions in liver, respectively.

Carboxylesterases, as a group, can hydrolyze a wide range of endogenous as well as exogenous ester substrates including simple aliphatic and aromatic esters, amides, monoacyl glycerols, acylcarnitines, acyl coA and cholesteryl esters [1, 2]. Their recognized physiological roles are to limit the activity of many ester drugs and to reduce the toxicity of pesticides to animals and to man [3].

Over the last two decades, six carboxylesterases have been purified from rat liver microsomes [2, 4, 5]. They have been named according to their isoelectric points (esterases' pI 5.0, 5.2, 5.6, 6.0, 6.2, 6.4). The isozymes share several molecular properties, for example, similar subunit weights, low carbohydrate content, one active site per protein subunit, presence of serine in the active site and selective sensitivity to inhibition by bis-4-nitrophenyl phosphate [1]. However, they possess different primary structures and substrate specificities toward xenobiotic drugs and lipids [5].

GSTs are a family of enzymes that catalyse the nucleophilic addition of the thiol of reduced glutathione to a variety of electrophiles [6]. In addition, GSTs bind with varying affinities a variety of hydrophobic compounds such as haem, bilirubin, polycyclic aromatic hydrocarbons and dexamethasone [7]. Similar to all other detoxification enzymes, GSTs enhance the aqueous solubility of drugs and thus facilitate their fecal and urinary excretion [8].

Several reports have shown that liver is the organ with the highest drug metabolizing activity [for example 1, 9]. The mammalian liver contains, in addition to its parenchymal cells, several types of non-parenchymal cells which line the sinusoids. The main non-parenchymal cells are the stellate (also called fat-storing cells, Ito cells or lipid-storing cells), endothelial and Kupffer cells [10]. The parenchymal cells constitute about 90% of the liver mass and represent about 65% of the cell number. The endothelial, Kupffer and stellate cells constitute about 60, 30 and 10%, respectively, of the non-parenchymal liver cells [11]. It has been shown that parenchymal cells are the main target for uptake and detoxification of drugs [12].

This study aimed to determine the distribution of carboxylesterases and GSTs between parenchymal and non-parenchymal cells in rat liver and also to

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† Abbreviations: GST, glutathione *S*-transferase; A−, vitamin A deficiency; A+, vitamin A-sufficient.

test whether the drug metabolizing activity of carboxylesterases and GSTs are affected by the vitamin A status of the animal. A− has been known to affect a wide variety of physiological functions as well as biological phenomena such as the immune response and antibody production [13], child morbidity and mortality [14], vision, reproduction, growth and cellular differentiation [15].

MATERIALS AND METHODS

Chemicals. Trypan blue, *o*-nitrophenyl acetate, *p*-nitrophenyl acetate, type I collagenase, Percoll, reduced glutathione, all-*trans* retinol and all-*trans* retinoic acid were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were from Fisher or Sigma.

Experimental design. Pregnant Sprague–Dawley rats were kept on chow diet until about 10 days of gestation. Mothers were then fed a vitamin A-free semisynthetic diet through pregnancy and lactation. This diet was composed of casein, corn starch, sucrose, vitamin mix (no vitamin A) and cotton seed oil. Pups were weaned at 21 days of age and divided into 3 groups, the first group (A−) was given vitamin A-depleted diet (0 μg vitamin A/g of diet), the second group ($A_{marg.}$) was given marginal vitamin A supplemented diet (0.18 μg vitamin A/g of diet) while the last group (A+) was given vitamin A-sufficient diet (4.0 μg vitamin A/g of diet) [13]. Some animals of the vitamin A-depleted group were repleted with a single intraperitonial injection of 20 μg of retinoic acid given 18 hr before sacrifice (A− + RA). All animals were allowed free access to food and water throughout the experiment. Rats were killed at 60 days of age and livers were taken for fractionation and enzyme assays. External signs of vitamin A deficiency (loss of appetite, loss of fur and slight abnormality in gait) started to appear in most of the vitamin A-deficient animals by the day 50. However, mortality was minimal (less than 10% of vitamin A-deficient animals). The vitamin A-deficient group tended to weigh slightly less than other groups. However, no significant difference was observed.

Separation of liver cells. The separation techniques were adopted from Matsuura [16]. Briefly, rat liver was perfused through the portal vein with 0.05% type I collagenase solution. The softened liver was then cut into small strips in 0.4% collagenase solution and incubated at 37° for 5 min. The solid scum of tissue was removed by a rough steel mesh and the remainder was filtered through cotton gauze in order to obtain a suspension of isolated cells. This suspension was centrifuged at a low speed of 75 *g* (600 rpm) for 5 min to separate the parenchymal from the non-parenchymal cells. Centrifugation was repeated twice to assure the precipitation of parenchymal cells. By centrifugation of the supernatant at 300 *g* (1500 rpm) for 3 min, the non-parenchymal cells were sedimented.

The sediment was then suspended in 10.57 mL of PBS buffer and mixed with 6.75 mL of Percoll and 0.68 mL of 10-fold concentration PBS. The resulting 38% Percoll cell suspension was centrifuged at 20,000 *g* (14,000 rpm) for 20 min. Stellate and endothelial cells formed a layer at the top of the tube whereas Kupffer cells formed a layer slightly above the red blood cells and debris which resided in the bottom of the tube. The layers were collected and the cells resuspended in PBS buffer. All fractions were subjected to identification by light microscopy and vitamin A fluorescence microscopy (cells were irradiated with UV at the wavelength of vitamin A excitation (325–345 nm) and the emitted fluorescence was detected) in addition to assessment of cell viability by the Trypan blue exclusion technique.

Enzyme assays. Total non-specific carboxylesterase activity was assayed colorimetrically at 420 nm by following the production of *o*-nitrophenol from *o*-nitrophenyl acetate [17]. The reaction mixture was formed of 2.7 mL of a solution containing 20 mM potssium phosphate buffer pH 7.4, 1 mM EDTA and 0.1% Triton-X 100, in addition to 0.25 mL of the enzyme fraction. After 10 min at 25°, the reaction was started by addition of 0.05 mL of 0.18 M *o*-nitrophenyl acetate in ice-cold methanol and followed for 10 min. A blank with no enzyme was run simultaneously to correct for spontaneous substrate hydrolysis.

Assay of GST activity was based on the reaction of *p*-nitrophenyl acetate with thiols to produce *p*-nitrophenol and acylated thiols [18]. All GSTs catalysed this reaction yielding *p*-nitrophenol quantitated directly by its absorbance at 400 nm. The assay solution included 1 mL of phosphate buffer containing 0.2 mM *p*-nitrophenyl acetate, 10 μL of 50 mM reduced glutathione solution pH = 7 and 20 μL of enzyme fraction. The rate of change of absorbance at 400 nm was recorded and a correction was applied for the non-enzymatic reaction.

RESULTS

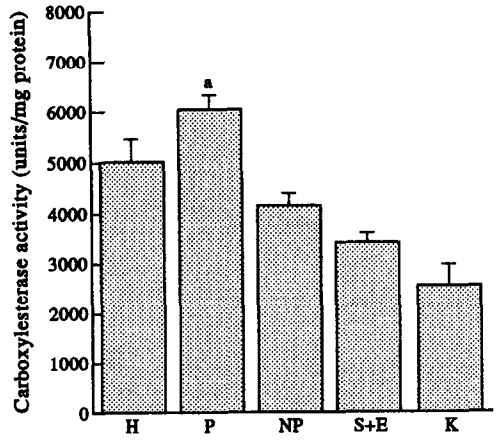
Distribution of carboxylesterase and GST activities in isolated rat liver cells of vitamin A-rich rats (A+)

Examination of liver cell fractions under light microscopy showed that in "parenchymal" fractions at least 95% of the cells are parenchymal cells and at least 90% of the cells are viable by Trypan blue exclusion. "Non-parenchymal" fractions were almost devoid of parenchymal cells and their cells had a viability over 90%. Examination of cells with vitamin A fluorescence microscopy revealed that about 90% of vitamin A fluorescence existed in "stellate + endothelial" fractions while the remaining fluorescence is found in the "parenchymal" fractions. "Kupffer" fractions were devoid of vitamin A fluorescence.

The results (Fig. 1) show that the activities of carboxylesterases and GSTs are mainly localized in parenchymal cells. However, activities were also present in non-parenchymal cells. Only the parenchymal cells showed considerable enrichment (higher specific activity) of carboxylesterases and GSTs over the whole homogenate. Kupffer cells showed the least enrichment of these activities among liver cells.

Effect of vitamin A status of rats on the total activities of carboxylesterases and GSTs in liver cells

As shown in Fig. 2, A− caused remarkably



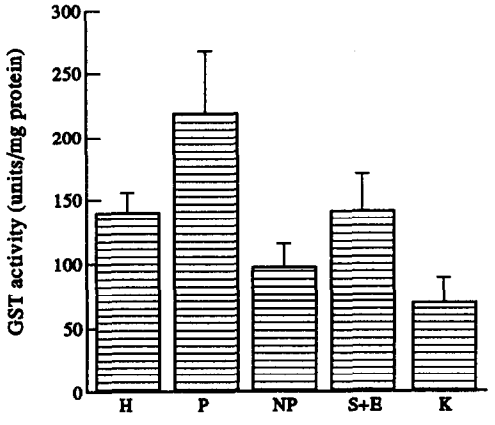
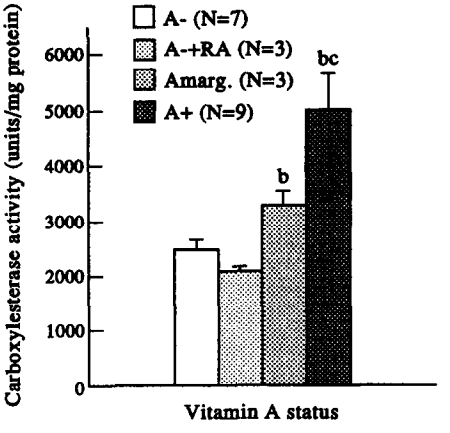


Fig. 1. Specific activities of carboxylesterases (top panel) and GSTs (bottom panel) in rat liver cells of A+ rats. Results are means ± SEM (N = 3). H, homogenate; P, parenchymal; NP, non-parenchymal; S + E, stellate cells and endothelial cells; K, Kupffer cells. Unit of activity is the change in absorbance at 400 nm/min × 10^{-3}. a, significance from non-parenchymal cells at $P < 0.05$.



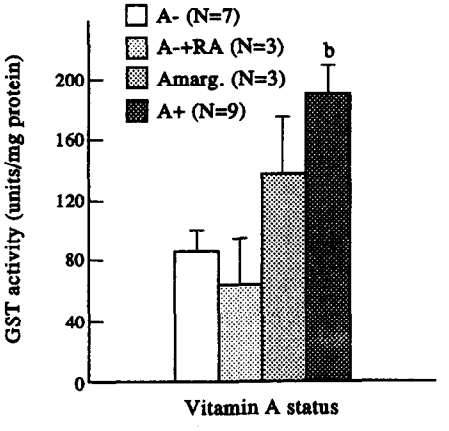
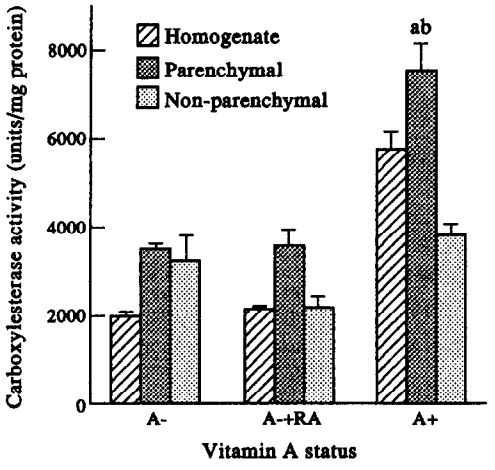


Fig. 2. Effect of A− on carboxylesterase (top panel) and GST (bottom panel) activities in rat liver homogenates. Results are means ± SEM. Number of animals are given in parentheses. A−, vitamin A-depleted rats; A− + RA, vitamin A-depleted rats given 20 μg retinoic acid 18 hr before sacrifice; A_{marg}, vitamin A-marginal rats; A+, vitamin A-sufficient rats. Units of activity are similar to Fig. 1 legend. b, significance from A− at $P < 0.05$. c, significance from A_{marg} at $P < 0.05$.

significant decreases in carboxylesterase and GST activities in rat liver homogenates as compared to A+ animals. This decrease was not ameliorated by administration of 20 μg retinoic acid 18 hr before sacrifice. Strong positive correlations ($r = 0.96$ for carboxylesterases and $r = 0.88$ for GSTs) between the activities of carboxylesterases and GSTs and the vitamin A status of the rats have been shown. Only female rats, similar in age and weight, were chosen for this experiment to abolish any effect on the enzyme activities other than the vitamin A status of the rats.

Effect of vitamin A status of rats on the distribution of carboxylesterase and GST activities in liver cells

In another set of experiments, the distribution of carboxylesterase and GST activities in parenchymal and non-parenchymal cells was studied in A−, A− + RA, and A+ groups (Fig. 3). The specific activities of carboxylesterases and GSTs in parenchymal cells of A+ animals were significantly higher than the corresponding activities in A− or A− + RA groups. This decrease in the activities of the A− and A− + RA groups was not significant in non-parenchymal cells. This indicated that the reduction in carboxylesterase and GST activities in liver homogenates of A− and A− + RA groups (shown in Figs 2 and 3) was mainly due to an effect on the activities of parenchymal cells rather than on the activities of non-parenchymal cells. This speculation was supported by the significant difference in specific activities of carboxylesterases and GSTs between parenchymal and non-parenchymal cells observed only in A+ but not in A− and A− + RA groups.



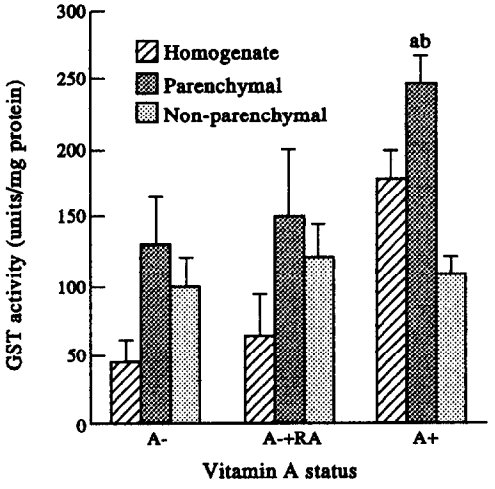
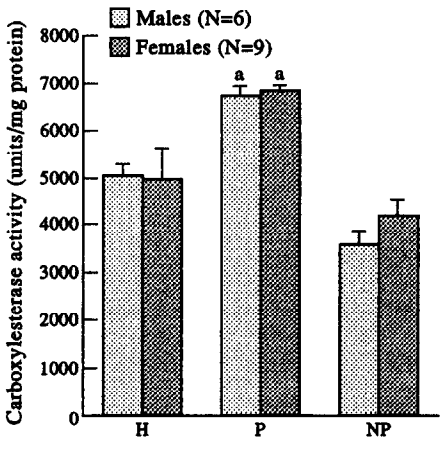


Fig. 3. Distributions of carboxylesterase (top panel) and GST (bottom panel) activities between parenchymal and non-parenchymal cells in rats with different states of vitamin A supplement. Results are means ± SEM. N = 3, 4 and 10 for A−, A− + RA and A+, respectively. A−, A− + RA and A+ are similar to Fig. 2 legend. a, significance from non-parenchymal cells at $P < 0.05$; b, significance from A− at $P < 0.05$.



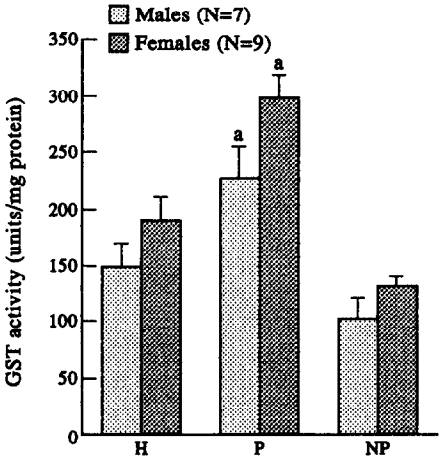


Fig. 4. Specific activities of carboxylesterases (top panel) and GSTs (bottom panel) in female and male A+ rat livers. Results are means ± SEM. Number of animals are shown in parentheses. H, homogenate; P, parenchymal cells; NP, non-parenchymal cells. a, significance from non-parenchymal cells at $P < 0.05$.

Effect of sex on the distribution of carboxylesterase and GST activities in rat liver cells

In the final set of comparative studies, the activities of carboxylesterase and GSTs in liver homogenates, parenchymal cells and non-parenchymal cells were compared between the males and females of A+ rats (Fig. 4). In all fractions, there was no significant difference between male and female rats with regard to carboxylesterase or GST activities. The difference in specific activity between parenchymal and non-parenchymal cells was similarly significant in both males and females.

DISCUSSION

Liver parenchymal cells have versatile functions when compared to the more specialized functions of Kupffer, endothelial and stellate cells. Both Kupffer and endothelial cells contain high specific activities of lysosomal enzymes and they constitute a coordinated defense system that protects parenchymal cells against injury [19]. Stellate cells represent the major lipid-storing cells especially for retinoids [20]. Thus, in agreement with the wide functions of parenchymal cells, this study showed that most of the carboxylesterase and GST activities are found in the parenchymal cells. In fact, since parenchymal cells represent about 90% of liver cell mass, a conclusion can be drawn that over 90% of carboxylesterase and GST activities are localized in the parenchymal cells, on an absolute basis. It is unclear, however, whether the presence of these activities in non-parenchymal cells has significant physiological function or just a limited defensive

mechanism for detoxification of some products that are generated in non-parenchymal cells *in situ*.

As the substrates used for the assay of carboxylesterases and GSTs are not specific for one of the isozymes of carboxylesterases or GSTs, it is therefore difficult to identify whether one or more isozymes are present in non-parenchymal cells. In fact, the low non-specific hydrolysis or conjugation observed in the non-parenchymal cells may even be a non-specific activity for other enzymes such as retinyl ester hydrolase(s) or cholesteryl ester hydrolase(s). Gausted *et al.* [12], in their study of the distribution of different carboxylesterase isozymes in liver cells, have shown that non-parenchymal cells are enriched only with the isozymes pI 6.2/6.4. These two closely related isozymes are more specific toward hydrolysis of lipid substrates such as palmityl coA, monoacylglycerols and retinyl palmitate. This finding addresses the question whether the presence of esterases 6.2/6.4 with their specific lipolytic activities in non-parenchymal cells is related to the specific function of stellate cells with their high lipid metabolizing activity. The lowest enrichment of carboxylesterase and GST activities observed in Kupffer cells agrees well with the more specialized function of Kupffer cells that depends on lysosomal enzymes.

The activities of drug metabolizing enzymes are generally influenced by several physiological and/or pathological conditions, for example, sex [1], obesity [21], diabetes and fasting [22], developmental stage [23], hypertension [23] as well as by administration of several drugs such as phenobarbitone [24] and glucocorticoids [7]. Therefore, many researchers have considered these enzymes as "adaptive" enzymes, responding to changes in homeostatic conditions in the animal, during both altered physiological and pathophysiological states. This postulation may explain the remarkable reduction in carboxylesterase and GST activities in A− (Figs 2 and 3) despite the absence of a clear link between carboxylesterase and GST activities and vitamin A metabolism. In fact, non-specific carboxylesterases have very low affinity for the hydrolysis of retinyl palmitate, the major physiological form of retinyl esters (only the isozymes p*I* 6.2/6.4 can hydrolyze retinyl palmitate [25]) and their contribution to the total hydrolysis of retinyl palmitate by liver subcellular fractions does not exceed 7% [25, 26]. In addition, vitamin A is not an immediate substrate for GSTs.

One explanation for this effect is that vitamin A might be a positive inducer of carboxylesterase and GST synthesis at the level of gene expression. However, this explanation does not comply with the observation that retinoic acid did not show any replenishment in carboxylesterase and GST activities due to vitamin A deficiency. Retinoic acid is known to be the most potent regulator of gene expression among retinoids [27]. The high positive correlations between carboxylesterases and GSTs and vitamin A status of the animals ($r = 0.96$ and 0.88, respectively) points to a direct link between the vitamin A status of the rats and the activities of drug metabolizing enzymes especially in parenchymal cells since the levels of carboxylesterase and GST activities in non-parenchymal cells are not well correlated to the vitamin A status (Fig. 3).

With regard to the effect of sex on the distribution and content of carboxylesterase and GSTs in liver cells, Mentlein and his coworkers [1] have previously reported on the influence of sex on the individual carboxylesterase isozymes. However, they did not describe any difference between male and female rats with regard total content, cellular distribution or subcellular localization of carboxylesterases. In this study, no significant difference was observed between male and female rats with regard to the total activities of carboxylesterases and GSTs in liver homogenates and cellular distribution of these enzymes between parenchymal and non-parenchymal cells.

This study opens up a new era of investigations to shed more light on the potential role of non-parenchymal cells in detoxification reactions and to elucidate the possible mechanisms for regulation of carboxylesterase and GST activities by vitamin A. Further studies are also required to detect whether all or specific isozymes of carboxylesterases and GSTs are sensitive to vitamin A deficiency and whether other drug metabolizing enzymes are similarly sensitive to vitamin A status of the animal.

Acknowledgements—Many thanks to Dr T. Matsuura for his kind technical advice and guidance for the preparation and identification of liver cell fractions. Best compliments to Dr Yehia Gad for his valuable advice.

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