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AGE-ASSOCIATED ALTERATIONS IN HEPATIC GLUTATHIONE-*S*-TRANSFERASE ACTIVITIES

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Abstract—Age-associated alterations of hepatic cytosolic glutathione-*S*-transferase (GST) activities towards sulfobromophthalein sodium tetrahydrate (BSP), styrene oxide (STOX), trans-4-phenyl-3-butene-2-one (PBO), 1,2-dichloro-4-nitrobenzene (DCNB), and 1-chloro-2,4-dinitrobenzene (CDNB) were investigated in Fischer-344 rats of both sexes with ages ranging from 1.5 to 28 months. The GST activities towards PBO and DCNB in male rats increased with age till 6–12 months when maximum values were attained, and then gradually decreased till 28 months when the values became the lowest. The GST activities towards STOX and BSP did not show any significant increase after 1.5 months and stayed at this level till 12 months, followed by a gradual decrease till 28 months when the values were the lowest.

In contrast, the GST activity towards CDNB in male rats did not show much of an age-associated alteration. Age-associated alterations in GST activities in females were much smaller than those observed in males. Sex differences in GST activities (significantly higher male values than female values) were observed with all the substrates examined at least at some time of the animal life.

The kinetic studies of GST activities indicated that alterations in the relative abundance as well as the total quantity of GST isozymes caused the substrate selective alterations of GST activities with age.

Senescence-associated alterations of the ability of the liver to metabolize and detoxify incoming xenobiotics (including drugs) are of importance from many points of view, such as geriatric pharmacology, oncology, toxicology and environmental health. In the past, age-associated alterations of monooxygenase activities (the phase I reaction of drug metabolism) have attained much more attention from investigators (see ref. [1] for review) than those of phase II reactions [2–5] of the detoxication of xenobiotics which are similarly important. Our investigations on hepatic microsomal monooxygenase activities have shown that the patterns of age-associated alterations of drug metabolizing activities are substrate specific and, probably, enzyme-species specific [6–8]. In order to clarify whether similar age-associated alterations occur for the activities of the phase II reactions, we studied one of the phase II reactions, glutathione-*S*-transferase (GST, EC 2.5.1.18) activities in relation to rat age.

MATERIALS AND METHODS

Chemicals

Sulfobromophthalein sodium tetrahydrate (BSP) was purchased from Aldrich Chemical Co. (U.S.A.); 1,2-epoxyethylbenzene (styrene oxide, STOX); benzalacetone (trans-4-phenyl-3-buten-2-one, PBO); 1,2-dichloro-4-nitrobenzene (DCNB); 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) were purchased from Wako Pure Chemical Ltd, Japan. Other chemicals for the measurement of enzyme activities were all of analytical grade.

Animals and tissue preparation

Specific pathogen-free Fischer-344 rats of both sexes were purchased at the age of 4 weeks from Japan Charles River Co. (Atsugi). They were raised in the SPF aging farm of the Tokyo Metropolitan Institute of Gerontology, with controlled lighting (08:00–20:00 hr), temperature (22 ± 2°), and humidity (50–60%). Acidified water (pH 2.5–3.0; residual chlorine, 10 ppm) and commercial rat pellets (CRF1, Oriental, Tokyo) were given *ad lib.*

Group of 1.5, 3, 6, 12, 24 and 28-month-old male and female rats, each consisting of at least five animals, were used. Livers were individually homogenized (1:3, w/v) in ice-cold 1.15% KCl, pH 7.0, with a Potter–Elvehjem homogenizer. Homogenates were centrifuged at 9000 *g* for 20 min and the supernatant was then centrifuged for 1 hr at 105,000 *g* to separate the cytosol fraction.

Assays for glutathione-S-transferase activities

Glutathione-*S*-transferase activities towards five different substrates, CDNB [9], DCNB [10], PBO [10], BSP [11] and STOX [9, 12] were assayed according to previously described methods with minor modifications. Routine incubation mixtures for GSH conjugations towards CDNB, DCNB and STOX contained hepatic cytosol fractions, 0.5 mM

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of GSH and 0.5 mM of one of the substrates in a 0.13 M phosphate buffer, pH 7.0 for CDNB, a 0.1 M phosphate buffer, pH 7.5 for DCNB, and a 0.13 M phosphate buffer, pH 8.0 for STOX. The final cytosolic protein concentrations in the incubation media were 10 μg/ml for CDNB, 0.25 mg/ml for DCNB, and 0.5 mg/ml for STOX. Total incubation volumes were always 1.5 ml. Mixtures were preincubated at 37° in a shaking water bath for 5 min for DCNB and STOX, and 3 min for CDNB. The reaction was started by adding the substrate; 15 mM STOX in EtOH 50 μl, 37.5 mM CDNB in DMSO 20 μl, except in the case of CDNB where the reaction was started by adding 30 μl of 25 mM GSH. The reaction took place at 37° in a shaking water bath for 5 min for DCNB and STOX, and 3 min for CDNB. Under these conditions, product formation was linear with respect to time and protein concentration. The reaction was stopped by the addition of 0.1 ml of a 33% aqueous trichloroacetic acid solution, followed by centrifugation at 800 *g* for 10 min. The concentrations of conjugated STOX [12], CDNB [9], and DCNB [10] were determined as previously described.

Routine incubation mixtures for GSH conjugations towards PBO and BSP contained 0.5 mg/ml cytosolic protein, 0.25 mM GSH, and 0.05 mM PBO in a 0.1 M phosphate buffer (pH 6.5) and 5 mM GSH and 0.03 mM BSP in a 0.1 M phosphate buffer (pH 8.2), respectively. Total incubation volumes were 3.0 ml. Mixtures without GSH were preincubated at 25° in a water bath for 3 min. The reaction was started by adding the GSH to the reaction mixture. The conjugate formation was directly determined by continuously measuring the absorbance change at 290 nm for PBO and at 330 nm for BSP. Appropriate controls in each experiment served as blanks and for determination of the spontaneous (non-enzymic) conjugation.

Varying substrate concentrations were used for the kinetic studies for glutathione-*S*-transferase activities towards PBO, DCNB and CDNB. Other conditions were not altered. Lineweaver–Burk plots were drawn for activities towards PBO and DCNB by using the least-square method. Kinetic parameters (V_{max}, K_m) were calculated by a computer (NEC, Japan) using a non-linear regression program (Simplex). In the case of GSH activity towards CDNB, curves were generated by a computer by using a non-linear least-square program, assuming two sets of Michaelis constants (K_{m1} and K_{m2}) and maximum velocities (V_{max1} and V_{max2}) and using the following equation:

$$V = \frac{V_{max1}S}{S + K_{m1}} + \frac{V_{max2}S}{S + K_{m2}}$$

where V is the overall reaction rate, and S is the substrate concentration. All determinations were performed at least in duplicate for each liver cytosol preparation from individual rats.

Statistical analysis

The significance for the difference between different animal groups was analyzed by Student's *t*-test (unpaired). P values lower than 0.05 were judged to be significant.

RESULTS AND DISCUSSION

Figure 1 indicates the age-associated alterations of GST activities towards five different substrates such as PBO (Fig. 1a), STOX (Fig. 1b), BSP (Fig. 1c), DCNB (Fig. 1d) and CDNB (Fig. 1e) in male and female rat liver cytosol preparations. The patterns of the age-associated alterations in GST activities in male rats varied depending on the substrate used, while alterations in female rats were much smaller. These patterns in males seem to be classified into the following three types: (I) The activities towards PBO and DCNB in males increased till maximum values which were attained at 6–12 months, and then gradually decreased till 28 months when the values became the lowest. (II) On the other hand, GST activities towards STOX and BSP did not show any significant increase after 1.5 months. The activity stayed at a high level till 12 months, and then gradually decreased till 24 or 28 months, the values being significantly lower than the corresponding 3-month-old values at 24 months or later. (III) In contrast to the above two patterns, the CDNB–GST activities did not show any significant decrease even at 28 months compared to younger values.

The pattern of age-associated alteration of STOX–GST activity is at variance with the observation of Birnbaum and Baird [13] who showed no decrease in STOX–GST activity with age in CFN rats. The reason for this discrepancy is not clear but it may be due to the difference in rat strain used. In contrast, Spearman *et al.* reported a significant decrease in STOX–GST activity [3], while no significant alteration with age was observed for CDNB–GST activity [4] in senescent Fischer-344 male rats. These observations are in good agreement with our study.

The results of kinetic analysis performed for GST activity towards PBO and DCNB in rats of different ages are shown in Fig. 2. For PBO–GST (Fig. 2a) and DCNB–GST (Fig. 2b) reactions, there was no age-associated alteration in K_m value, while the V_{max} was the largest at 12 months, indicating that the age-associated alterations of GST activities towards PBO and DCNB were due to the changes in enzyme content. On the other hand, the Lineweaver–Burke plot for CDNB–GST activity was not linear and the curve was best approximated by assuming two sets of Michaelis constants (K_{m1}, K_{m2}) and maximum velocity (V_{max1}, V_{max2}) (Fig. 3). Neither K_{m1} nor K_{m2} significantly altered with age, the overall average values being 0.055 ± 0.0049 mM for K_{m1} and 2.39 ± 0.206 mM for K_{m2} (mean ± S.E.). On the

Fig. 1. Age-associated changes in glutathione-*S*-transferase activity towards *trans*-4-phenyl-3-buten-2-one (PBO, Fig. 1a), stylene oxide (STOX, Fig. 1b), bromosulfophthalein (BSP, Fig. 1c), 1,2-dichloro-4-mitrobenzene (DCNB, Fig. 1d), and 1-chloro-2,4-dinitrobenzene (CDNB, Fig. 1e), in male and female rat liver cytosol. All values are given as means of product formation in μmoles/min/mg protein ± S.E. for aging livers of both sexes from seven rats individually assayed, at least in duplicate. —●— GST activity in male rats, ---○--- GST activity in female rats. *P < 0.05, **P < 0.01, significantly lower than corresponding 6-month-old values. ★P < 0.05, ★★P < 0.01, significantly lower than corresponding male values.

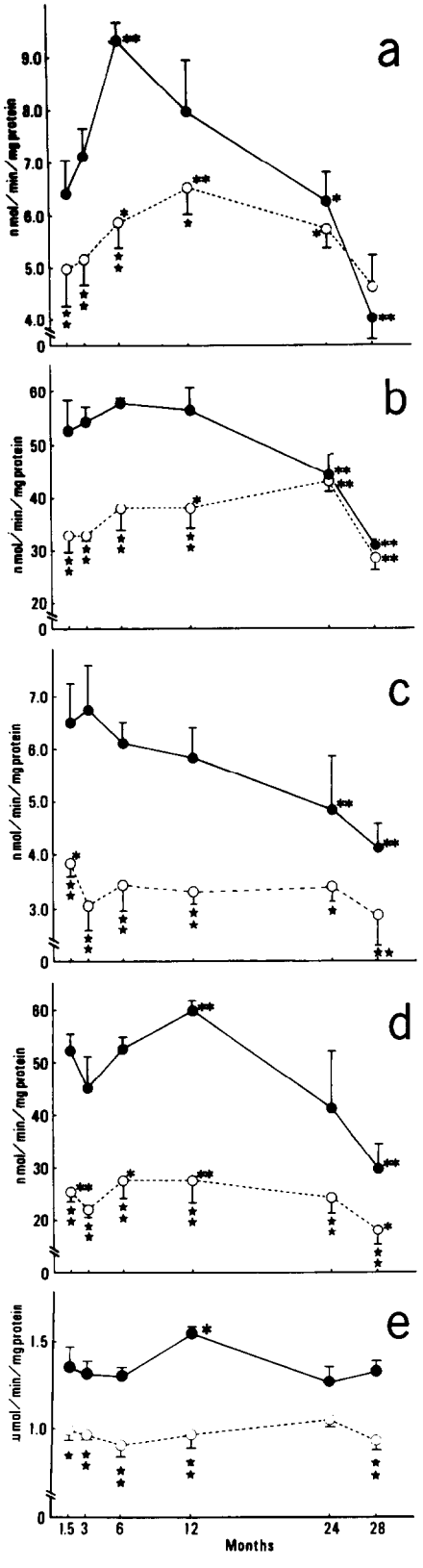

Fig. 1.

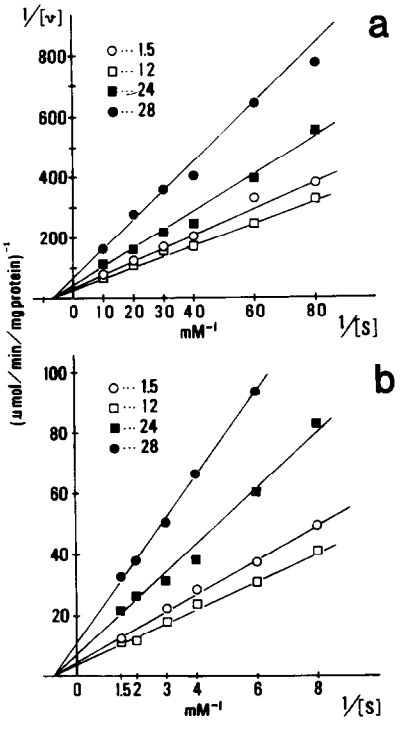

Fig. 2. Lineweaver–Burk plots showing the effect of substrate concentration on the reaction rate of aging rat liver glutathione-*S*-transferase activity using trans-4-phenyl-3-buten-2-one (PBO, Fig. 2a), 1,2-dichloro-4-nitrobenzene (DCNB, Fig. 2b). 1.5 (○), 12 (□), 24 (■) and 28 (●) month-old male rat cytosols were used in these experiments.

other hand, the V_{max1} tended to decrease but V_{max2} tended to increase with age (Fig. 4).

The existence of the multiple species of GST with overlapping substrate specificity is well known. These species of GST are usually classified into A–C and B groups [14]. An A–C group is distinguished from the B group by its high activity with DCNB. CDNB is a common substrate for all forms of GST,

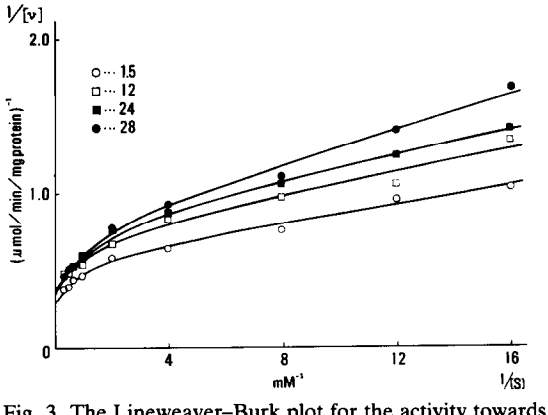

Fig. 3. The Lineweaver–Burk plot for the activity towards 1-chloro-2,4-dinitrobenzene (CDNB). Symbols are the same as in Fig. 2.

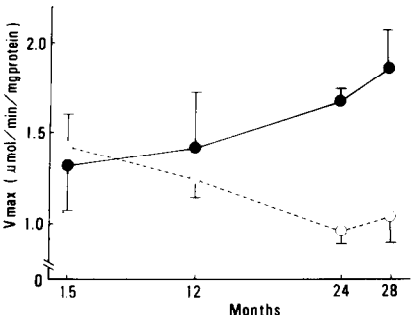

Fig. 4. Age-associated alterations in maximum velocities of the two different families of enzymes engaging in glutathione-*S*-transferation towards 1-chloro-2,4-dinitrobenzene. The values of V_{max1} (---○---) and V_{max2} (—●—) obtained in Fig. 3 were plotted against the age of the rats.

although transferase B is less efficient in transferring GSH towards CDNB as compared to A–C group enzymes [14]. Thus, the decrease in V_{max} for DCNB–GST and PBO–GST activities and unaltered K_m with age may be due to the parallel decrease in glutathione-*S*-transferases of the A–C group. Similarly, the decrease in V_{max1}, for CDNB–GST activity and unaltered K_{m1} which is much smaller than K_{m2}, with age may be due to the decrease in A–C group. On the other hand, the unaltered K_{m2} value and an increase in V_{max2} for CDNB–GST with age may be explained as the result of the increase in transferase B compensating for the age-associated decline of CDNB–GST activity due to the decrease in the A–C group. Thus, the data of the kinetic study on CDNB (Figs 3 and 4) may be best interpreted to mean that the relative abundance of species of transferase enzymes engaging in GSH conjugation towards CDNB, altered with age.

Our kinetic studies are partially supported by the observation of Kitahara *et al.* [2] who found an increase in glutathione-*S*-transferase B with age in rat livers using immunological techniques.

Spearman *et al.* [4] reported that an apparent K_m for CDNB–GST in male rat liver increased with old age. This can be well explained by our results, because increased contribution of transferase B with a larger K_m value, to this reaction would result in increased overall K_m value in senescent animals. However, this cannot be explained by their own purification study: these authors further observed in a recent study [5] an increase in transferase A and a decrease in transferase B in senescent Fischer-344 rats. This should result in decrease in overall apparent K_m value due to the increased contribution of a smaller K_m enzyme in the overall reaction. The reasons for the discrepancies between two studies of Spearman *et al.* [4, 5] or between their purification study [5] and our present observation are not clear. Except for these differences, these previous studies [2, 4, 5] seem to agree with the conclusion of our present study that the substrate selective alterations of GST activities with age are due to the alterations in total quantity as well as in the relative abundance of the multiple species of glutathione-*S*-transferase in the cytosol of liver cells.

Finally, we limited the above discussion to changes in male activities. As is clear from the results, the age-associated changes in GST activities are quite minor in female rat livers regardless of the substrate used. Thus, the generalization for the trend of age effect on these cytosolic enzyme activities should be carefully refrained as we emphasized in previous studies for microsomal enzyme activities [6–8].

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