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EFFECT OF THE AGING PROCESS ON THE GENDER AND PHENOBARBITAL DEPENDENT EXPRESSION OF GLUTATHIONE *S*-TRANSFERASE SUBUNITS IN BROWN NORWAY RAT LIVER

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Abstract—The effect of age, gender and phenobarbital treatment on the hepatic cytosolic glutathione *S*-transferase subunit composition was studied in Brown Norway rats. Affinity chromatography followed by reversed phase HPLC was used in order to separate the various glutathione *S*-transferase subunits. Corresponding steady-state mRNA levels were measured by Northern Blot analysis using cDNA clones hybridizing to mRNA encoding glutathione *S*-transferase subunits 1/2, 3/4 and 7, respectively. In all the age groups studied (15, 25, 53, 99, 112 and 136 weeks) the total amount of glutathione *S*-transferase protein was in untreated rats significantly higher in males (132 μg/mg cytosolic protein) than in females (91 μg/mg cytosolic protein) and significant gender dependent differences in the subunit composition were demonstrated. Aging seemed to be of minor importance in untreated as well as in phenobarbital treated rats. Under control conditions, the subunit composition of male rats between 15 and 136 weeks old consisted of 28, 12, 11 and 49% of subunits 1, 2, 3 and 4 respectively and of female animals of the same age groups of 38, 26, 7 and 30%, respectively. In all the age groups studied phenobarbital administration (45 mg/kg body weight, i.p., once a day for 7 days) doubled total glutathione *S*-transferase protein in both genders and affected the subunit composition in a significant way, emphasizing the already existing differences between genders. Subunits 1, 2 and 3, especially, were increased in male rats in comparison to females resulting in the observation that levels of glutathione *S*-transferase subunits studied became higher in males than in their female counterparts. The HPLC results were confirmed by steady-state mRNA analysis. In untreated rats, higher levels of mRNA encoding glutathione *S*-transferase subunits 1/2 and 3/4 were present in male than in female livers. Phenobarbital treatment increased mRNA levels in both genders. Subunit 7 was never detected. These effects were demonstrated in both young and old rats.

Susceptibility to drugs and foreign chemicals increases in conjunction with the aging process [1–3]. Although studies on the functional alteration of the human liver with age can only be carried out by indirect pharmacokinetic approaches, direct experimental work is possible with aging animals. From a review [4] of the many studies carried out with rodents of different ages it is known that the ability to remove various compounds, e.g. drugs, from the body decreases with age and that this process is gender dependent. Phase I biotransformation reactions, in particular mixed-function oxidase activities, are affected by the aging process [4–6]. Changes in phase II biotransformation reactions seem to be smaller but considerably less data are available [4] especially for rat cytosolic glutathione *S*-transferase (GST¶), (EC 2.5.1.18) [5–12].

GST consists of a family of isoenzymes capable of conjugating glutathione with a wide range of endogenous and exogenous electrophiles [13]. Some isoenzymes have a Se independent glutathione peroxidase activity which plays an inhibitory role on overall lipid peroxidation preventing eventual damage to membranes [14]. Since lipid peroxidation occurs during the aging process, changes in GST activity and in the expression of its isoenzyme profile may be of importance.

The rat cytosolic GSTs are homo- and heterodimers of 11 different subunits [15] which, on the basis of present evidence involving DNA and amino acid sequences, enzymatic properties and immunological cross-reactivity, have been classified into four families namely, the α, μ, π and θ families [16, 17]. In liver tissue the major subunits are 1, 2 and 3, 4 which belong to the α and μ family, respectively [18]. Subunit 7, absent in adult liver parenchymal cells, has only been reported in fetal liver [19], in early stages of hepatocarcinogenesis [20] and in cultures of adult rat hepatocytes [21, 22]. Data concerning subunit 7 as a function of aging are completely lacking.

In a recent work it has been shown that the GST activity in liver from Brown Norway rats changes significantly as a function of age and seems to be gender dependent [23]. Phenobarbital (PB), a well-known GST inducer [24, 25], was used as a reference

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¶ Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; PB, phenobarbital.

compound in order to study, in both genders, the response capacity to xenobiotics as a function of age. It was found to exert a significant effect [23].

In order to determine whether such changes affect the GST isoenzyme pattern with age and which isoenzymes are involved, liver cytosolic GST subunit patterns of male and female Brown Norway rats from 11 to 144 weeks old were analysed and their response to PB treatment was investigated. Some of the results were confirmed by Northern blot analysis using cDNA clones, hybridizing to mRNA, encoding GST subunits 1/2, 3/4 and 7, respectively.

MATERIALS AND METHODS

Animals. Inbred Brown Norway (BN/BiRij) rats were obtained from the TNO Institute for Experimental Gerontology, Rijswijk, The Netherlands. They were kept under "clean, conventional" conditions as described by Hollander [26]. Six age groups were studied: 15, 25, 53, 99, 112 and 136 weeks old in the case of control rats and 11, 26, 52, 104, 126 (females), 132 (males) and 144 (males) weeks old in the case of PB treated animals. Treated rats were given a daily dose of 45 mg PB/kg body weight by intraperitoneal injection for 7 days and 24 hr after the last injection, livers were removed. These conditions were previously determined as being suitable for young and old rats [23].

Liver and body weights of all the animals were measured and the liver weight/body weight ratio was calculated.

Materials. 1,2-Dichloro-4-nitrobenzene (DCNB) and trifluoro acetic acid were obtained from Merck-Schuchardt (Darmstadt, Germany), 1-chloro-2,4-dinitrobenzene (CDNB) from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and reduced glutathione from Boehringer Mannheim (Germany). Sodium phenobarbital was purchased from Siegfried S.A. (Zofingue, Switzerland), heparin from Novo Industries (Copenhagen, Denmark) and nembutal from Ceva (Brussels, Belgium). Acetonitrile, HPLC-grade, was from Carlo Erba (Milano, Italy) and the Nick translation kit and [^{32}P]dCTP were obtained from Amersham International (Amersham, U.K.). All other compounds were readily available commercial products.

Preparation of liver cytosol. Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). After intravenous injection of heparin (1000 I.U./mL) the liver was removed and homogenized (1/3, w/v) in ice-cold 22 mM sodium phosphate buffer pH 7.0, containing 1 mM ethylenediamine tetra acetate and 0.25 mM phenylmethyl-sulfonylfluoride.

Liver cytosol was prepared from the 9000 *g* supernatant by centrifugation at 105,000 *g* for 70 min.

Purification of GST isoenzymes. The purification step was carried out immediately after the preparation of the cytosol using a shortened version [21] of the method of Vander Jagt *et al.* [27].

Analysis of GST subunits by reversed phase HPLC. Separation and quantification of GST subunits were carried out by HPLC on a 10 × 0.8 cm Waters μBondapak C-18 reversed phase column in a Z-module using Waters system (Milford U.S.A.) according to Ostlund-Farrants *et al.* [28]. The solvents were water (A) and acetonitrile (B) each containing 0.06% (w/w) trifluoroacetic acid. The samples were injected at 36% B.

During a run, a linear gradient was used from 36 to 53% B over 60 min with a flow rate of 1.5 mL/min. Detection was carried out at 214 nm. GST isoenzyme 5-5 is not retained by the glutathione affinity column [29] and was therefore not measured here.

GST subunits, separated by HPLC, were identified by comparing their retention times with those of purified GST. Quantitative data were obtained by recovery of the respective subunits from HPLC and their molar extinction coefficients at 214 nm, as given by Ostlund-Farrants *et al.* [28].

Northern blots. Total RNA was isolated from small pieces of PB-treated and untreated livers by using the procedure of Chirgwin *et al.* [30] and was stored at −80°. For Northern blot analysis, 20 μg RNA were subjected to electrophoresis in a denaturing formaldehyde-agarose gel (1.5%) and transferred onto nylon filters. These filters were prehybridized and hybridized with nick-translated probes (400–600 10^6 cpm/μg DNA). pGSTr 155 [31] is complementary to mRNA coding for GST subunit 1 and cross-hybridizes with subunit 2 mRNA; JT9L (Pemble and Taylor, unpublished results) is

Table 1. Liver weight/body weight ratio as a function of age in male and female rats with and without PB treatment

Gender	Age (weeks) Control	Age (weeks) PB	Liver weight/body weight (%) Control	Liver weight/body weight (%) PB
Female	15	11	3.77 ± 0.35*†	4.60 ± 0.26[a]
	25	26	3.10 ± 0.20*‡§	4.30 ± 0.10[c]
	53	52	3.13 ± 0.12†‖	4.43 ± 0.23[c]
	99	104	3.57 ± 0.06	4.63 ± 0.42[b]
	112	126	3.87 ± 0.12‡	19.9 ± 0.32[a]
	136	—	3.90 ± 0.14§‖	—
Male	15	11	3.13 ± 0.21	4.62 ± 0.23[c]
	25	26	2.80 ± 0.17	3.93 ± 0.15[c]
	53	52	2.90 ± 0.28	4.23 ± 0.12[b]
	99	104	3.17 ± 0.31	4.27 ± 0.29[b]
	112	132	2.93 ± 0.25	4.25 ± 0.07[c]
		144	—	4.36 ± 0.21

The results are shown as means ± SD (N = 3).

Statistical differences as a function of age are indicated at the level 0.05. Only in untreated females were some differences observed:

* 15–25 weeks.
† 15–53 weeks.
‡ 25–112 weeks.
§ 25–136 weeks.
‖ 53–136 weeks.

As a function of gender no significant differences were observed with the exception of control rats of 112 weeks old ($P < 0.05$) and PB treated rats of 26 weeks old ($P < 0.05$).

As a function of treatment, all the PB treated rats showed a significantly higher liver weight body weight ratio than the control rats and this was found for both genders ([a], $P < 0.05$; [b], $P < 0.01$; [c], $P < 0.001$).

complementary to subunit 3 mRNA and cross-hybridizes with subunit 4 mRNA; the nomenclature "mRNA encoding GST subunits 1/2 and 3/4" is therefore used. pGSTr 7 is complementary to subunit 7 mRNA [32]. After hybridization, filters were washed, dried and autoradiographed at −80°.

Protein determination. Proteins were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

Statistics. The results were analysed by ANOVA tests with two or three variables wherever suitable. Where a significant difference was found, one-way analysis of variance with one variable was applied [33].

RESULTS

Liver weight/body weight ratio in control and PB treated rats

In Table 1 the liver weight/body weight ratios in control and PB treated rats as a function of age are shown. Although the liver and body weights of both genders in both groups significantly increased as a function of age, in control rats no consistent significant change occurred in the liver weight/body weight ratio. On the contrary, administration of PB had a significant effect on the ratio in all the age groups involved: an average increase of 30% in females and of 40% in males was observed. The liver weight/body weight ratio is usually of importance with regard to the metabolic capacity of the liver, since many drugs are dosed based on body weight.

Cytosolic protein concentration in control and PB treated rats

In Table 2 the cytosolic protein concentration in male and female treated and control rats are displayed as a function of age.

Aging seemed to have no consistent significant effect on the cytosolic protein content. In addition, no significant effects of gender and PB treatment were observed.

GST subunit pattern in control rat liver

A typical HPLC separation obtained with untreated female liver samples is displayed in Fig. 1.

In Fig. 2 the quantitative GST subunit patterns of control female (a) and male (b) rat liver are summarized for the age groups studied. From a general statistical analysis with two variables (gender and age), it appears that only gender had a significant effect on the GST subunit composition. As a function of age, no statistical significant changes were observed. For male rat liver the total GST protein content was 132 μg/mg cytosolic protein in all the age groups involved; in the case of females 91 μg/mg cytosolic protein was measured. More specific statistical analysis with one variable (gender) revealed that subunits 3 and 4 were significantly different between male and female rats ($P < 0.01$), male rats (all age groups combined) having 160 and 130% more of subunits 3 and 4, respectively, than female rats.

Subunits 1 and 2 were augmented in male and

Table 2. Cytosolic protein concentration of male and female rats with and without PB treatment as a function of age

Gender	Age (weeks) Control	PB	Cytosolic proteins (mg/mL) Control	PB
Female	15	11	17.4 ± 1.9	16.2 ± 0.5
	25	26	16.5 ± 1.4	17.2 ± 0.8
	53	52	21.5 ± 2.2*†	18.3 ± 0.9
	99	104	14.9 ± 2.6*	17.2 ± 1.6
	112	126	16.8 ± 1.2	19.9 ± 5.0
	136	—	16.0 ± 0.3†	—
Male	15	11	20.7 ± 1.4	16.0 ± 2.5
	25	26	17.3 ± 1.1	17.8 ± 1.1
	53	52	22.8 ± 2.9	19.0 ± 0.9
	99	104	18.3 ± 2.7	20.0 ± 0.7
	112	132	21.5 ± 1.2	16.0 ± 2.4
	—	144	—	19.9 ± 3.4

The results are shown as means ± SD (N = 3).

The effect of PB treatment on the liver weight has been taken into account during the preparation of the cytosol making the data for controls and PB treated rats comparable.

Statistical analysis: Effect of age: for controls, significant difference ($P < 0.05$) between (*) 53–99 and (†) 53–136 weeks; for PB treated rats, not significant (NS).

Effect of gender: NS for controls and PB treated rats.

Effect of treatment: NS for all age groups and both genders.

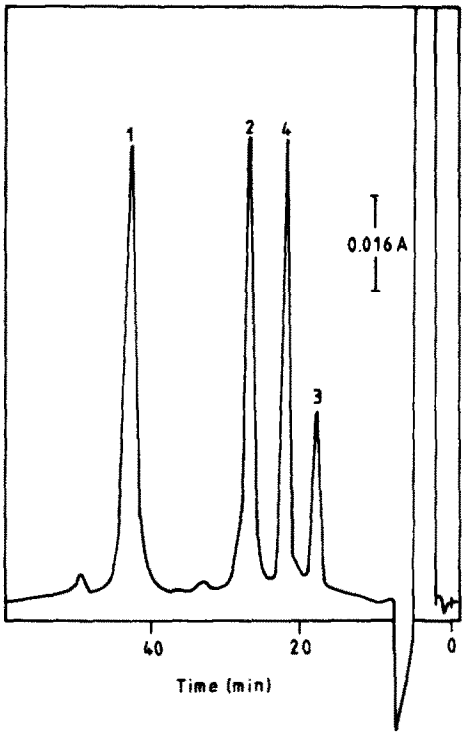

Fig. 1. Typical separation of female rat liver GST subunits by reversed phase HPLC.

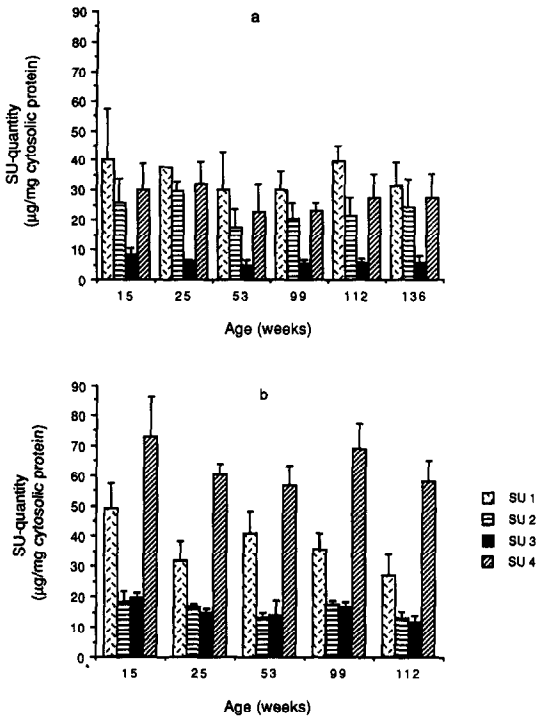

Fig. 2. GST subunit composition of female (a) and male (b) control livers. The results are presented as a function of the age of the animals. Mean ± SD is shown (N = 3); SU_i, subunits of GST.

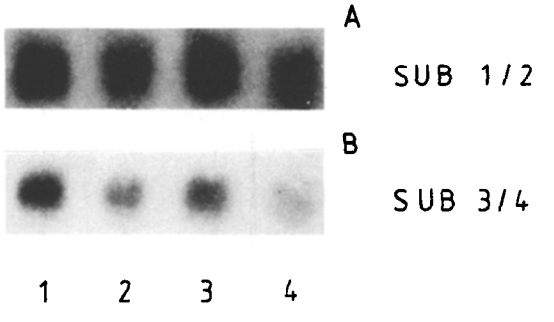

Fig. 3. Northern blot analysis of mRNA encoding GST subunits 1/2 (A) and 3/4 (B) in liver of male and female rats of 15 and 25 weeks old. RNA (20 µg) was loaded in each lane: lanes 1,3: male liver of 15 and 25 weeks, respectively; lanes 2,4: female liver of 15 and 25 weeks, respectively.

female rats, respectively but this was not statistically relevant. Subunit 7 protein was not detected in either gender in any of the age groups studied.

Steady-state mRNA analysis (Fig. 3B) showed clearly more mRNA encoding GST subunits 3/4 in male than in female liver samples, in good agreement with corresponding GST protein content. For subunits 1/2 it also appeared that more mRNA was present in male liver although the difference was

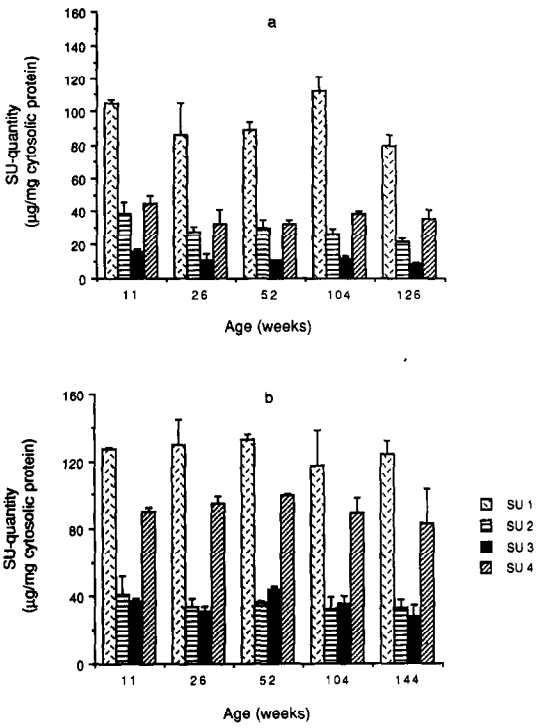

Fig. 4. GST subunit composition of female (a) and male (b) PB treated livers. The results are presented as a function of age of the animals. Results are means ± SD (N = 3).

less clear as shown in the Northern blot, than for subunit 3/4 (Fig. 3A). This observation is in accordance with the corresponding GST-protein content (Fig. 2). In Fig. 3, results are shown for the age groups of 15 and 25 weeks, being representative for all the age groups studied. mRNA encoding GST subunit 7 was not observed in male or female rat liver samples, this being the case for all the age groups examined (results not shown here).

Effect of PB on the GST subunit pattern of rat liver

In Fig. 4 the GST subunit patterns of PB treated female (a) and male (b) rats are shown for the various age groups studied. A general statistical analysis with three variables (age, gender and PB-treatment) points to a significant effect of PB treatment and gender but not age.

The total GST protein content for all age groups combined was 289 µg/mg and 172 µg/mg cytosolic protein representing total induction factors of 2.2 and 1.9 for males and females, respectively. The level of each subunit was significantly higher in male than female rat liver ($P < 0.05$) and this was true for all the age groups studied. The levels of subunits 1, 2, 3 and 4 were 30, 20, 200 and 150%, respectively, higher in male than in female PB treated animals.

When the subunit pattern of untreated animals was compared with that of PB treated rats it appeared that PB exerted a significant effect on the GST subunit patterns of both genders.

In Table 3 an overview of the percentage increases of the various GST subunits after PB treatment is shown. For both genders the highest values were

Table 3. Percentage increase of rat liver GST subunits after PB treatment

Gender	GST subunits (% increase) 1	2	3	4
Female	173*	25	94*	36
Male	242*	127*	129*	44*

Since it was shown clearly that there were no significant differences between the various age groups for either gender, the results represent means of all the age groups involved.

The results obtained after PB treatment have been expressed against the values of control animals and the percentage increase is shown here for each subunit.

Significant differences are indicated: * significancy at the 95% level ($P < 0.05$) for the mean value and for all the individual age groups involved.

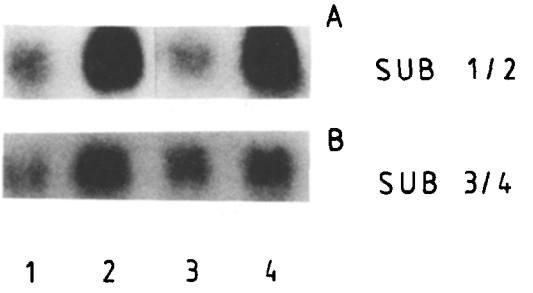

Fig. 5. Northern blot analysis of mRNA encoding GST subunits 1/2 (A) and 3/4 (B) in control and PB treated rat liver. RNA (20 μg) was loaded in each lane: lanes 1, 3: control male liver samples of rats of 99 and 112 weeks old, respectively; lanes 2, 4: PB treated male liver samples of rats of 104 and 132 weeks old, respectively.

Table 4. Comparison of the sex dependent differences in GST subunit levels between control and PB treated rat liver

GST subunits	Control	PB
1	♀ = ♂	♀ < ♂ (30)*
2	♂ < ♀ (45)	♀ < ♂ (20)
3	♀ < ♂ (160)*	♀ < ♂ (200)†
4	♀ < ♂ (130)*	♀ < ♂ (150)‡

The percentage difference between the individual subunit levels in male and female livers is shown in brackets.

Since it was shown clearly that there were no significant differences between the various age groups for either gender, the results are the means of all age groups.

Significant differences are indicated and refer to the mean values and all the individual age groups involved: * $P < 0.005$; † $P < 0.01$ and ‡ $P < 0.001$.

observed for subunits 1 and 3. Also important to notice is the fact that, whereas in male control rats the level of subunit 2 tended to be lower than in female animals, after PB treatment it became 20% higher (see Table 4). It is clear that PB had a more pronounced effect on the GST subunit pattern in male than in female liver. When the gender dependent differences observed between untreated and PB treated rats were compared (Table 4) it became obvious that PB had a specific effect in enlarging the already existing gender differences. Steady-state mRNA analysis (Fig. 5) again confirmed the HPLC results just mentioned. Northern blot analysis on RNA isolated from control and PB treated rat liver of all age groups and of both genders revealed that after PB treatment the mRNA levels encoding GST subunits 1/2 and 3/4 in male (Fig. 5) and in female livers (results not shown) had increased and gender dependent differences were also observed. Again, a clear increase was observed after PB treatment for all age groups and for both genders. mRNA encoding GST subunit 7 was not observed in either gender in any of the age groups studied.

DISCUSSION

Drugs can be metabolized by the liver via conjugation or phase I, followed by phase II reactions.

The effect of aging on phase I reactions, mostly cytochrome P450 dependent, has been studied extensively in rats and from review of the literature it is clear that aging has a pronounced effect [4]. The cytochrome P450 content and the NADPH-cytochrome *c* reductase activity decrease while no changes are observed for cytochrome b_5. Most cytochrome P450 dependent monooxygenase activities decrease in male rats but not in females [4, 6].

Glutathione conjugation, an important phase II system, has been less studied as a function of aging and usually only GST activities have been measured. Almost no attention has been paid to the involvement of the GST isoenzyme pattern [4, 8, 9, 11, 12, 23].

Changes in the GST isoenzyme profile, however, can change the susceptibility to xenobiotics since it has been established that the various GST isoenzymes have different functions and different metabolic capacities towards various compounds [18, 34–36]. Besides their catalytic activity in glutathione conjugation, mostly leading to detoxification, subunits 1, 2, 5 and 7 have a Se independent peroxidase activity resulting in inhibition of lipid peroxidation [14]. Lipid hydroperoxides formed by the effects of reactive oxygen species can undergo very damaging, metal catalysed, decomposition to free radicals, electrophilic and mutagenic hydroxyalkenals [37]. The reduction of these hydroperoxides by GST isoenzymes to form fatty acid alcohols has a drastic inhibitory effect on overall lipid peroxidation, a process which may be increased during aging [38].

From previous work in Brown Norway rats [23] it appeared that not only aging but in an even more pronounced way, gender and PB treatment have a significant effect on the ratio of the total GST activity (towards CDNB) to the more specific GST activity of the isoenzymes 3–3 and 3–4 (towards DCNB), possibly pointing to changes in the GST isoenzyme profile. PB was used here as a reference drug in

order to study the response capacity of the liver GSTs to xenobiotics in general.

From the results reported in this study concerning the effect of the factors aging, gender and PB treatment on the GST subunit profile, it appears that, in agreement with previous results [23], gender and PB treatment are more important variables than is the aging process.

Concerning the effect of aging, it should be mentioned that a trend towards decreasing GST proteins as a function of age is found when the total amount of GST subunits is taken into account for male rats. In female animals, an identical shaped curve is observed as obtained for the general GST activity [23] when the total GST proteins are expressed as a function of age. No statistically relevant data, however, could be obtained for the individual GST subunits in conjunction with the aging process. To our knowledge, only one study deals with the effect of age on the GST profile of control rat liver [39]. The author concludes that aging is an important factor although the argument provided is rather poor since only one male rat per age group was examined and a different technique was used.

It should be noted that the results obtained here are expressed against cytosolic proteins. It was found in this study (Table 2) that aging seemed to have no consistent significant effect on the cytosolic protein content of female and male liver. This finding is in agreement with the data of Van Bezooijen *et al.* [40], reporting a constant protein synthesis in freshly isolated hepatocytes of female BN/BiRij rats, aged 3–12 and 18–24 months. Only in more advanced ages were sharp increases noticed which were not detected in our oldest age groups. In addition, no significant effects of gender and PB treatment were observed.

As far as gender is concerned, a significant effect was observed in all age groups studied: the level of total GST protein was higher in male than in female rat liver and subunits 3 and 4 represented nearly 60% of the total amount in male compared to only 35% in female livers. Agreement with the literature is not evident since only a limited number of experiments directly dealing with the GST isoenzymes have been carried out with female rats and most results have been obtained using SDS–polyacrylamide gel electrophoresis resulting in percentage subunit patterns [41, 42]. Hales and Neims [24], however, using immunoprecipitation with anti-GST-B antibody, demonstrated higher 3–3, 3–4 and 4–4 activities in males and higher 1–1, 1–2 and 2–2 activities in females which is in good agreement with the quantitative data described in this study.

From measurements of the GST activity towards different substrates such as CDNB, DCNB [24, 41, 43, 44], cumene hydroperoxide [41], sulfbromophtalein [44] and styrene oxide [45] it is clear that gender differences do exist on the level of the GST isoenzymes but what the differences are and how they evolve with aging and PB exposure is still unknown.

When PB treatment is considered, it appears that significant changes occurred in the GST subunit patterns of both genders for all age groups involved. Aging seemed to be of less importance in PB treated rats as far as the individual GST subunits were concerned although in female rats a Pearson correlation coefficient of 0.712 was measured ($P < 0.001$) for the total amount of GST proteins as a function of age. For males no such correlation was found. Gender, on the contrary, was an important factor: when rats were exposed to PB all GST subunits in both genders increased, in particular, subunits 1 and 3, whose increase was more pronounced in males than in females. The same was true for subunit 2. PB exposure seemed to have a selective effect, emphasizing the already existing differences under control conditions.

From a review of the literature by Igarashi and Satoh [46] it is clear that GST activity in adult rats is induced by PB treatment and this inducibility is much greater in males than in females. 1–2 type GSTs are more affected than 3–4 type isoenzymes [41] which is in good agreement with our findings.

Steady state mRNA analysis confirmed some of the results mentioned, namely, more mRNA encoding GST subunits 1/2 and 3/4 was present in male than in female liver in all age groups, pointing to important sex differences. mRNA encoding GST subunit 7 was never present, showing that under control conditions and during PB exposure no *de novo* expression of an extra fetal type GST isoenzyme with high peroxidase activity seems to be necessary. In previous work also, mRNA encoding GST subunit 7 was never detected in the liver of control adult rats [47].

For all the age groups involved, PB treatment increased mRNA levels encoding subunits 1/2 and 3/4 in both genders. Also clear sex differences were observed that were consistent with previously published data for young adult rats [48]. It has been reported that PB exposure increases the transcription rate of GST subunits 1 and 3, especially, while subunit 2 gene transcription is barely affected [48]. These changes result in an increase in the relative concentration of subunits 1 and 3 in comparison with that of subunits 2 and 4 [25].

In conclusion, all these results point to the observation that changes of the GST subunit profile are not responsible for the increased susceptibility to xenobiotics observed in conjunction with aging as far as rats are concerned. Phase I reactions seem to be of much more importance.

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