Bioelectrochemistry and Bioenergetics, 18 (1987) 247–256
A section of *J. Electroanal. Chem.*, and constituting Vol. 232 (1987)
Elsevier Sequoia S.A., Lausanne – Printed in The Netherlands

1058 — INDUCTION OF SELENIUM-GLUTATHIONE PEROXIDASE BY STIMULATION OF METABOLIC HYDROGEN PEROXIDE PRODUCTION *IN VIVO* *

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SUMMARY

Selenium-glutathione peroxidase (Se-GPx) provides a major detoxication pathway for hydrogen peroxide produced in the cytosolic and mitochondrial compartments of rat liver. The aim of this study was to assess the influence of an increase in N-demethylase (cytochrome P_{450}-dependent) or monoamine oxidase (MAO) activity on Se-GPx biosynthesis *in vivo*. Male Sprague–Dawley rats injected i.p. with ethylmorphine (60 mg/kg in physiological saline, twice a day) exhibited an increase in hepatic Se-GPx activity from the second day of intoxication, with a maximal increase of approximately 2-fold after 4 days, whereas a similar introxication with β-phenylethylamine (50 mg/kg) did not induce liver Se-GPx in spite of a long-lasting malonaldehyde overproduction. Ethylmorphine did not induce liver catalase. No induction of Se-GPx was observed in females injected with ethylmorphine. When compared with males, such females have a 2- to 3-fold higher hepatic Se-GPx activity, and they do not exhibit a marked diurnal cycle of hepatic GSH concentration as males do. A decrease in hepatic GSH concentration was observed after the second daily injection of ethylmorphine in both males and females, but this decrease was more pronounced and prolonged in females.

Our observations are consistent with stimulation of Se-GPx biosynthesis in male rat liver by a cytochrome P_{450}-dependent, but not by an MAO-dependent H_2O_2 production *in vivo*. In females, a major pathway for ethylmorphine hepatic detoxication is glutathione-dependent, but may not involve coupling of N-demethylase and Se-GPx activities.

* Presented at the Bioelectrochemical Society Meeting on "Formation and Reactions of Peroxides in Biological Systems", Obernai, 22–24 October 1986.

0302-4598/87/$03.50

INTRODUCTION

Using glutathione (GSH) as a cofactor, glutathione peroxidase reduces hydrogen peroxide to water [1] and organic hydroperoxides to the corresponding alcohols [2], according to the following stoichiometry:

$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O \quad (1)$$

or

$$ROOH + 2\ GSH \rightarrow GSSG + ROH + H_2O \quad (2)$$

Through this detoxication pathway, selenium-glutathione peroxidase has been thought to account for the essentiality of selenium as a trace nutrient in mammals [3]. This ubiquitous enzyme is present in the cytosolic and mitochondrial compartments of most cells, where it works in synergy with superoxide dismutases and vitamin E. The current concept is that cell protection by glutathione peroxidase may be critical in situations of oxidative stress.

The most common form of selenium-glutathione peroxidase (Se-GPx) is a tetramer composed of identical subunits, each of which contains an essential atom of selenium at the active site [4,5]. Inducibility is a common feature of many protective enzymes. Induction of selenium-glutathione peroxidase has been reported in the lung during prolonged hyperoxia [6,7]. Although the availability of selenium is a limiting factor in the biosynthesis of Se-GPx, induction of Se-GPx would be expected in situations where intracellular hydroperoxides are overproduced. Indeed, 30–50% increases in Se-GPx activity have been reported in rat liver following intoxication by CCl_4 [8], autoxidized lipids [9,10] or 4-chloro-2-methylphenoxyacetic acid [11]. The latter compound is a herbicide known to induce peroxisomal proliferation and hydrogen peroxide accumulation.

Intracellular degradation of hydrogen peroxide by catalase is limited by its compartmentation into peroxisomes [12] and its low affinity for the substrate [13]. Under physiological conditions where the H_2O_2 concentration is low and the glutathione concentration is high, the Se-GPx pathway accounts for most of the cytosolic and mitochondrial H_2O_2 degradation [14].

The aim of this study was to assess the influence of a well-defined metabolic stimulation of hydrogen peroxide production on the biosynthesis of Se-GPx in the liver and brain. For this purpose, repeated intoxications with either ethylmorphine or β-phenylethylamine were used.

In microsomes, ethylmorphine is demethylated by a cytochrome P_{450}-dependent N-demethylase, with stoichiometric production of hydrogen peroxide and formaldehyde [15]. Formaldehyde is not detected in ethylmorphine-treated hepatocytes, due to its rapid oxidation to formate in a glutathione-dependent pathway where S-formylglutathione initially formed by formaldehyde dehydrogenase is then hydrolysed by S-formylglutathione hydrolase [16]. Under normal conditions, H_2O_2 released in the cytosolic compartment, from ethylmorphine oxidative N-demethylation, is decomposed by Se-GPx and not by catalase [17].

As a substrate of monoamine oxidase, β-phenylethylamine is a source of

mitochondrial hydrogen peroxide [18] which should also be decomposed by Se-GPx in the mitochondria [19].

EXPERIMENTAL

The animals used in this study were Sprague–Dawley rats (Iffa-Credo, l'Arbresle, France) weighing 200 g at the beginning of the experiment. Unless otherwise mentioned, all animals were fed a rat chow diet *ad libitum*. In all experiments of intoxication by means of i.p. injections of either ethylmorphine or β-phenylethylamine (solution in physiological saline), control rats were similarly injected with physiological saline alone.

Time course of enzyme induction during ethylmorphine intoxication

The rats were repeatedly exposed to toxic doses of ethylmorphine by means of two daily i.p. injections of either 60, 75 or 90 mg of ethylmorphine per kg of body weight, the first one around 10 a.m. and the second one 8 h later. Unless otherwise mentioned, daily i.p. injections were stopped after 5 days. The activities of selenium-independent and selenium-dependent glutathione peroxidases as well as that of catalase were then measured in whole tissue extracts of liver and brain at various time-points.

Time course of liver glutathione concentration after ethylmorphine injections

The rats received two successive i.p. injections of 60 mg of ethylmorphine per kg of body weight, the first one at 10 a.m. and the second one at 6 p.m. Rats were killed at time zero, 20, 40 and 60 min following each injection, and reduced glutathione concentrations were measured in whole liver homogenates.

Intoxication with β-phenylethylamine

Male rats were repeatedly exposed to toxic doses of β-phenylethylamine by means of daily i.p. injections of 50 mg/kg body weight. Glutathione peroxidase activity and malonaldehyde content were then measured in whole liver homogenates and/or mitochondrial liver extracts at various time-points.

Preparation of tissue samples

The animals were killed by decapitation at various time-points, following diethyl ether anaesthesia and cerebral perfusion through the left heart with 50 cm^3 physiological saline containing 10 units/cm^3 heparin and 0.5% pyrogallol as an antioxidant. The brain and liver were immediately excised.

For measurements of enzyme activities and total proteins in whole tissue extracts, aqueous tissue homogenates were made at 4°C with a Teflon pestle homogenizer in

50 m*M* Tris–HCl, 0.1 m*M* EDTA, 0.1% lubrol, pH 7.6 (7:1, *V*: *w*), and aliquots of low-speed supernatants were used. For measurement of reduced glutathione content, aqueous tissue homogenates were similarly made in 5% sulphosalicylic acid (10:1, *V*: *w*) and protein precipitation was achieved by centrifugation at 5000 *g* for 10 min. For measurement of glutathione peroxidase activity in mitochondrial extracts, the latter were obtained as described by Eichberg *et al.* [20].

Enzyme assays

Glutathione peroxidase activity was measured by a modification of the method of Paglia and Valentine [21], at 0.25 m*M* GSH, pH 7.6, 37°C, and using 0.2 m*M* hydrogen peroxide or cumene hydroperoxide as substrate in two separate assays. Catalase activity was measured by monitoring the disappearance of 10 m*M* H_2O_2 at 240 nm [22] in 50 m*M* Tris–HCl, 0.1 m*M* EDTA, 0.1% lubrol, pH 7.4, and 37°C; the presence of 0.1% lubrol allowed satisfactorily linear initial rates in tissue extracts to be obtained.

Glutathione-S-transferase activity was measured spectrophotometrically at pH 6.5 and 25°C, using 1 m*M* 1-chloro-2,4-dinitrobenzene as substrate in the presence of 1 m*M* reduced glutathione [23]. N-Demethylase activity was measured at pH 7.4 and 37°C by monitoring formaldehyde formation [24], using 4 m*M* aminopyrine or 10 m*M* ethylmorphine as substrate in two separate assays [25].

Other assays

Reduced glutathione was measured as the monobromobimane adduct, by reverse phase h.p.l.c. on a 10 μm C18 column (4.6 × 250 mm), with fluorimetric detection, as described by Anderson [26]; reduced cysteine was added in the 5% sulphosalicylic acid homogenates as an internal standard. Malonaldehyde was measured as the optical density (535 nm) of the thiobarbituric acid-adduct, on aliquots of tissue aqueous homogenate (10/1, *V*/*w*) made in 1.15% KCl [27]; malonaldehyde-(bis)-diethylacetal was used as an external standard. Total proteins were measured by the dye-binding assay of Bradford [28], using bovine serum albumin as a standard. For statistical evaluation of the data, paired comparisons were made using Student's *t*-test.

RESULTS

As shown in Fig. 1A (lower curve), repeated i.p. injections of male rats with 75 mg of ethylmorphine per kg of body weight caused a significant increase ($p < 0.01$) in hepatic Se-GPx activity, which was maximal (approximately +50%) after 4–5 days of intoxication. On day 11, *i.e.* 5 days after injections of ethylmorphine had been stopped, glutathione peroxidase activity was still above the control values ($p < 0.01$).

Since about 30% of the rats died during the first 2 days of intoxication, it was

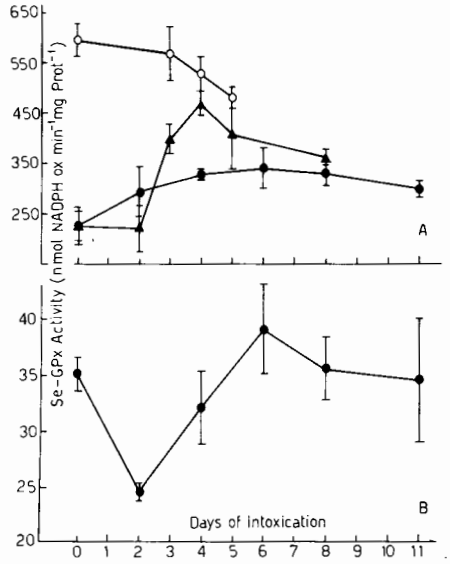

Fig. 1. Time course of Se-GPx activity mean ± s.d., $N \geqslant 4$ during intoxication with ethylmorphine. Two i.p. injections per day, as described in the Experimental section. (A) Se-GPx activity in the liver; male rats, 2×75 mg kg^{-1} day^{-1} (●) or 2×60 mg kg^{-1} day (▲); female rats, 2×90 mg kg^{-1} day^{-1} (○). (B) Se-GPx activity in the brain of male rats, 2×60 mg kg^{-1} day^{-1}.

important to check that the apparent increase in Se-GPx activity was not due, in part, to a better survival of the rats already having a higher level of enzyme activity at the beginning of the experiment. This was done by decreasing the injected dose of ethylmorphine. As shown in Fig. 1A (middle curve), repeated injections of 60 mg of ethylmorphine per kg caused a much larger increase in hepatic Se-GPx activity. This increase was maximal (approximately 100%) after 4 days of intoxication and could not be improved upon longer times of intoxication. There was no increase in selenium-independent glutathione peroxidase activity.

When females were similarly intoxicated by repeated injections of 60 mg of ethylmorphine per kg, no significant induction of Se-GPx activity was observed (data not shown) and this level of intoxication caused a smaller number of deaths than in males. As shown in Fig. 1A (upper curve), increasing the dose of injected ethylmorphine up to 90 mg/kg caused a marked decrease in hepatic Se-GPx activity in females. At the same time, a much higher incidence of female deaths (approximately 30%) was observed.

The time course of Se-GPx activity in the brain of male rats injected with 60 mg of ethylmorphine per kg is shown in Fig. 1B. Although ethylmorphine intoxication caused a significant decrease ($p < 0.01$) in brain Se-GPx activity after 2 days of intoxication, a return to normal values was observed after 4 days and no significant induction of Se-GPx activity could be obtained upon further intoxication. Similarly,

TABLE 1

Selenium-glutathione peroxidase activity in the liver and brain of male rats injected i.p. with β-phenylethylamine (50 mg/kg)

	Liver [a]		Brain [a]	
	Control	Treated	Control	Treated
Homogenate	254 ± 19	201 ± 48	13.0 ± 1.5	10.8 ± 2.4
Mitochondria	87 ± 15	107 ± 17	19.8 ± 4.0	18.3 ± 1.97

[a] nmol H_2O_2 reduced min^{-1} mg^{-1} protein at 0.25 m*M* GSH, 37 °C and pH 7.6; $N = 4$; differences are not significant.

no induction of brain Se-GPx activity was obtained with higher doses of ethylmorphine in either male or female rats (data not shown). Also, ethylmorphine intoxication did not cause any significant change in either the liver or brain catalase activity in rats of either sex.

As shown in Table 1, repeated i.p. injections of male rats with 50 mg of β-phenylethylamine per kg caused no significant induction of selenium-glutathione peroxidase activity in either whole aqueous homogenates or mitochondrial extracts of liver and brain. At this level of intoxication, more than 30 % of the rats died and a persistent malonaldehyde overproduction was observed in the liver (see Table 2). On the contrary, such a persistent lipid peroxidation was not observed with ethylmorphine-treated rats.

The effect of ethylmorphine injections (60 mg/kg) on the GSH concentration in the liver of males and females is shown in Fig. 2. In male rats, the first (10 a.m.) and second (6 p.m.) i.p. injections caused a small but significant decrease in the GSH concentration ($p < 0.01$) after 40 min, while a return towards control values was observed by 60 min. In females, the first injection (10 a.m.) did not cause any significant change in the liver GSH concentration, while the second one (6 p.m.) caused a marked decrease which was maximal by 20 min (-25%, $p < 0.01$), with no recovering trend by 60 min.

The diurnal cycle of the GSH concentration in the liver of non-intoxicated rats is shown in Fig. 3. A marked diurnal cycle was observed in the liver of males, with a 30% decrease between 9 a.m. and 6 p.m. ($p < 0.01$). Although a similar trend was

TABLE 2

Malonaldehyde concentration in liver extracts of rats injected i.p. with ethylmorphine or β-phenylethylamine

	Control [a]	Treated [a]
Ethylmorphine	0.77 ± 0.11	0.82 ± 0.11
β-phenylethylamine	0.77 ± 0.11	1.72 ± 0.22 [b]

[a] nmol MDA mg^{-1} protein ($N = 4$).
[b] $p < 0.001$.

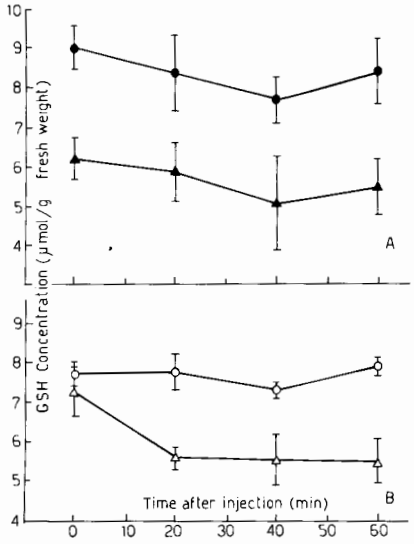

Fig. 2. Time course of liver GSH concentration mean ± s.d., $N \geqslant 6$ after intoxication with ethylmorphine. Intraperitoneal injections of 60 mg of ethylmorphine per kg. (A) Male rats after first injection at 10 a.m. (●) and after second injection at 6 p.m. (▲). (B) Female rats after first injection at 10 a.m. (○) and after second injection at 6 p.m. (△).

apparent in the liver of females, the concentration range between extreme values was much smaller and a clear-cut demonstration of a small diurnal cycle would require more data points.

Finally, to assess the possibility that sex differences in glutathione consumption could reflect differences in glutathione-dependent pathways for ethylmorphine

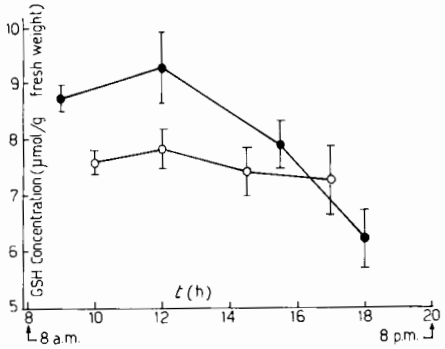

Fig. 3. Diurnal cycle of liver GSH concentration mean ± s.d., $N \geqslant 6$ in non-intoxicated rats. Male rats (●); female rats (○).

TABLE 3

Glutathione-S-transferase and N-demethylase activities in the liver of Sprague–Dawley rats

Substrate	GSH-S-transferase [a]		N-Demethylase [b]	
	Males	Females	Males	Females
Chloro-dinitro-2,4-benzene	5.9 ± 0.3	10.0 ± 0.8 [c]	–	–
Aminopyrine	–	–	0.40 ± 0.08	0.27 ± 0.04 [c]
Ethylmorphine	–	–	0.24 ± 0.04	0.24 ± 0.07 [d]

[a] μmol of substrate consumed min^{-1} g^{-1} fresh weight.
[b] μmol of formaldehyde produced min^{-1} g^{-1} fresh weight.
[c] $p < 0.01$, when compared with males.
[d] Not significant, when compared with males.

degradation, glutathione-S-transferase and N-demethylase activities were measured in the liver of untreated rats. As shown in Table 3, glutathione-S-transferase activity, when measured with chloro-dinitro-2,4-benzene as a substrate, was indeed significantly higher in females ($p < 0.01$). Although aminopyrine N-demethylase activity was significantly lower in females, there was no significant sex difference in ethylmorphine N-demethylase activity.

DISCUSSION

Our starting hypothesis was that intoxication with a chemical that would be catabolized with stoichiometric production of hydrogen peroxide could result in the induction of selenium-glutathione peroxidase biosynthesis if the latter was actually a major protective enzyme against hydrogen peroxide toxicity *in vivo*. This study has demonstrated that ethylmorphine does induce the biosynthesis of Se-GPx in the liver of male rats, while there is no significant induction of selenium-independent glutathione peroxidase or catalase. These findings are in contrast with a previous paper [29] in which repeated administration of aminopyrine was reported to cause an 80% increase in selenium-independent glutathione peroxidase, whereas the increase in Se-GPx activity was significant only after 1 day of intoxication.

The absence of induction of Se-GPx in the brain upon systemic intoxication with ethylmorphine is probably due to its very low content in cytochrome P_{450}, but it may also be due to a rather inefficient crossing of the blood–brain barrier.

Interestingly, ethylmorphine caused much less lipid peroxidation than β-phenylethylamine. Yet this MAO-induced oxidative stress did not result in Se-GPx induction. Hence, cytosolic hydrogen peroxide may be a better inducer of Se-GPx than either hydrogen peroxide or lipid peroxides of mitochondrial origin.

The reason why ethylmorphine does not cause induction of the Se-GPx activity in the liver of females is not clear. When measured with ethylmorphine as a substrate, no sex difference in N-demethylase activity was found. One important point is that basal Se-GPx activity is two to three times higher in the liver of females. This suggests that Se-GPx may be more limiting in males.

Another possible factor of sex differences is the glutathione status of the liver. The marked diurnal cycle that we observed in male rats is consistent with the results of previous investigators [30] where the diurnal cycle of liver GSH showed a maximum around noon and a minimum around 8 p.m. More unexpectedly, this study shows that females do not exhibit a marked diurnal cycle of hepatic GSH concentration.

The time courses of glutathione consumption following intoxication with ethylmorphine are also markedly different in males and females. In this connection, it is interesting to note the higher activities of glutathione-S-transferase in the liver of females. A degradation pathway involving covalent binding of GSH to an oxidatively activated derivative of morphine has been reported [31.32] and may also exist with ethylmorphine. Hence, in females, a glutathione-dependent pathway for ethylmorphine hepatic detoxication exists, but the major pathway may not involve coupling of N-demethylase and Se-GPx activities.

One may wonder whether the induction reported here does not reflect a particular relationship between selenium-dependent and cytochrome P_{450}-dependent pathways. Selenium has been shown to induce essential enzymes of haem metabolism [33] and selenium deficiency depresses a number of cytochrome P_{450}-dependent enzymes [34], the latter effect being more important in males [35]. However, a detailed analysis of this process led to the conclusion that a selenium mediator other than Se-GPx was involved in the regulation of microsomal enzyme synthesis [36,37].

Finally, an interesting question relates to the source of selenium that was used by the rats for incorporation at the active site of induced GPx. Preliminary experiments with rats fed a Se-deficient diet suggest that a substantial part of this selenium is of endogenous origin and that an extra-hepatic selenium pool may be used for this purpose. These results underline important questions about the mechanisms of adaptation of tissues to an acute oxidative stress.

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