Oxidation of glutathione by free radical intermediates formed during peroxidase-catalyzed *N*-demethylation reactions

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Received 22 August 1983

Horseradish peroxidase-catalyzed *N*-demethylation of aminopyrine and dimethylaniline results in generation of free radical intermediates which can interact with glutathione (GSH) to form a glutathione radical. This can either dimerize to yield glutathione disulfide or react with O_2 to form oxygenated products of glutathione. Ethylmorphine is not a substrate in the peroxidase-mediated reaction, and free radical intermediates which react with GSH, are not formed from aminopyrine and dimethylaniline when the horseradish peroxidase/H_2O_2 system is replaced by liver microsomes and NADPH. Therefore, it appears unlikely that formation of free radical intermediates can be responsible for the depletion of GSH observed during *N*-demethylation of several drugs in isolated liver cells.

Glutathione *Thiol oxidase* *γ-Glutamyl transferase* *Tubular epithelium* *Renal cell* *Plasma membrane*

1. INTRODUCTION

The *N*-demethylation of various drugs by isolated hepatocytes has been found to be associated with a decrease in intracellular glutathione (GSH) level which may lead to depletion of the cells of GSH under certain experimental conditions [1,2]. This was first interpreted as an effect of stimulated glutathione peroxidase activity caused by increased intracellular H_2O_2 generation as a result of drug-stimulated production of superoxide anion radicals ($O_2^{\cdot-}$) by the cytochrome P450 monooxygenase system [2]. Stimulation of glutathione peroxidase activity has been found to be associated with an efflux from the hepatocytes of a minor fraction of the glutathione disulfide (GSSG) formed in the reaction with isolated hepatocytes [1,2] as well as with the perfused rat liver [3]. However, enhanced production of oxygen radicals by the monooxygenase is not the predominant mechanism responsible for GSH consumption during *N*-demethylation of ethylmorphine and aminopyrine in isolated hepatocytes [4].

N-Demethylation reactions can also be catalyzed by peroxidases such as horseradish peroxidase (HRP) and prostaglandin synthase (PGS); the peroxidase-mediated reactions involve the formation of free radical intermediates as indicated by ESR spectroscopy [5,6]. Moreover, cytochrome P450 can also function as a peroxidase in presence of organic hydroperoxides or H_2O_2, and this hemoprotein has been found capable of catalyzing a variety of reactions, including *N*-demethylations, by a peroxidative mechanism [7]. A radical pathway of *N*-demethylation, analogous to that proposed for other hemoprotein–hydroperoxide systems, has been suggested for the metabolism of aminopyrine by cytochrome P450 peroxidatic activity [8].

Oxidation of GSH to GSSG occurs when the thiol is present during peroxidase-mediated drug oxidation; e.g., the PGS-linked metabolism of paracetamol and *p*-phenetidine [9,10]. This has been ascribed to the interaction of GSH with a radical intermediate ($R^{\cdot}$), formed by one-electron oxidation of the drug substrate, resulting in the formation of a thiyl radical ($GS^{\cdot}$) and regeneration

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Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

of the drug substrate; GSSG formation can subsequently occur by dimerization of two GS· radicals:

$$R^{\cdot} + GSH \longrightarrow RH + GS$$

$$GS^{\cdot} + GS^{\cdot} \longrightarrow GSSG$$

Thus, it appears that oxidation of GSH to GSSG during drug biotransformation may not only be a consequence of stimulated glutathione peroxidase activity but can also occur by direct interaction of GSH with free radical intermediates formed from various drug substrates. The present study was designed to investigate whether non-enzymatic oxidation of GSH by this latter mechanism may contribute to the consumption of glutathione observed during *N*-demethylation of drugs in isolated liver cells.

2. METHODS

Fed, male Sprague-Dawley rats (200–250 g body wt) which had been given sodium phenobarbital in the drinking water (1 mg/ml) for 7 days, were used throughout this study.

Hepatocytes were isolated by collagenase perfusion of the liver as in [11]. Liver microsomes were isolated as in [12].

Hepatocyte incubations were performed using rotating, roundbottom flasks under a 93.5% O_2–6.5% CO_2 atmosphere at 37°C, and at 10^6 cells/ml in a Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid). Incubations with microsomes were in 100 mM Tris–HCl (pH 7.4) containing EDTA (5 mM), at 37°C, and 1 mg protein/ml; the reaction was initiated with cumene hydroperoxide (0.2 mM) or NADPH (1 mM). Incubations with horseradish peroxidase, finally, were also performed in 100 mM Tris–HCl (pH 7.4) containing EGTA (5 mM), but at 25°C; [HRP] was 0.1 mg/ml, and the reaction was started by the addition of H_2O_2 (1 mM).

Protein concentration was assayed as in [13], formaldehyde according to [14], and GSH and GSSG as in [15]. Oxygen uptake was measured by monitoring oxygen tension in a closed cell fitted with a Clark type oxygen electrode (Rank Brothers, Bottisham) at a polarizing voltage of −0.6 V and 25°C, in a final volume of 2 ml.

3. RESULTS

Incubation of isolated hepatocytes from phenobarbital-treated rats with aminopyrine, dimethylaniline or ethylmorphine, caused a marked decrease in intracellular GSH concentration (fig.1). This was most prominent in the presence of ethylmorphine; after 30 min incubation with this drug only 25% of the original GSH level remained. The effect was dependent on cytochrome P450-linked metabolism of the substrates, since no GSH depletion was observed in the presence of the cytochrome P450 inhibitor metyrapone (not shown).

To investigate whether formation of free radical intermediates from the three drugs could be responsible for the consumption of GSH during their metabolism in isolated hepatocytes, the formation of formaldehyde from each of the drugs was assayed in three different experimental systems: horseradish peroxidase + hydrogen peroxide (HRP/H_2O_2), liver microsomes + cumene hydroperoxide, and liver microsomes + NADPH. Aminopyrine and dimethylaniline were metabolized by all three systems, HRP/H_2O_2 being most effective (table 1). Ethylmorphine, on the other hand, was only metabolized at a significant rate by liver microsomes + NADPH.

Addition of GSH caused a marked inhibition of the HRP/H_2O_2-catalyzed demethylation of aminopyrine and dimethylaniline (table 1). This in-

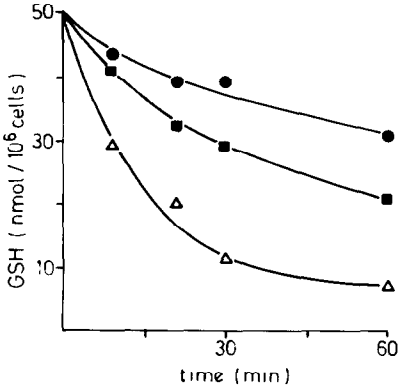

Fig.1. Decrease in intracellular GSH level during *N*-demethylation of various drugs in isolated hepatocytes. Hepatocytes were incubated as in section 2 in the presence of: aminopyrine, 5 mM (●—●); dimethylaniline, 5 mM (■—■); or ethylmorphine, 5 mM (△—△). One experiment typical of 5.

Table 1

Rate of *N*-demethylation of various drugs in different experimental systems
Effect of GSH

	Aminopyrine (5 mM)		Dimethylaniline (5 mM)		Ethylmorphine (5 mM)	
	Formaldehyde (nmol.min^{-1}.mg protein^{-1})					
	− GSH	+ GSH (1 mM)	− GSH	+ GSH (1 mM)	− GSH	+ GSH (1 mM)
HRP/H_2O_2	233.3 ± 24.0	75.5 ± 3.9	232.8 ± 41.8	83.0 ± 11.5	2.2 ± 1.1	1.4 ± 0.7
Microsomes/Cumene-OOH	50.5 ± 3.6	39.3 ± 0.9	72.8 ± 2.6	76.0 ± 0.8	5.7 ± 0.5	7.3 ± 0.8
Microsomes/NADPH	22.1 ± 1.6	24.4 ± 2.0	26.9 ± 1.7	28.4 ± 4.2	19.2 ± 2.0	16.3 ± 1.0

Incubations were performed as in section 2. Values represent means ± SE of 3–4 different expt

Table 2

Oxidation of GSH to GSSG during drug *N*-demethylation in different experimental systems

	Aminopyrine (5 mM)	Dimethylaniline (5 mM)	Ethylmorphine (5 mM)
	GSSG formation (nmol.min^{-1}.mg protein^{-1})		
HRP/H_2O_2	84.5 ± 4.1	35.6 ± 2.4	2.5 ± 1.1
Microsomes/Cumene-OOH	0.6 ± 0.4	11.5 ± 2.7	0.5 ± 0.3
Microsomes/NADPH	1.5 ± 0.2	2.7 ± 0.2	1.7 ± 0.4

Incubations were performed in presence of 1 mM GSH as in section 2. Values represent means ± SE of 3–4 different expt

Table 3

Effect of GSH on O_2 uptake during HRP/H_2O_2-catalyzed *N*-demethylation of various drugs

Substrate	O_2 uptake (nmol.min^{-1}.0.1 mg protein^{-1})	
	− GSH	+ GSH (1 mM)
H_2O_2 (1 mM)	6.8 ± 1.0	6.8 ± 0.9
+ Aminopyrine (5 mM)	50.0 ± 5.2	368.0 ± 35.3
+ Dimethylaniline (5 mM)	6.7 ± 0.5	245.0 ± 30.2
+ Ethylmorphine (5 mM)	5.9 ± 0.5	5.9 ± 0.5

Incubations were performed as in section 2. Values represent means ± SE of 3 different expt

hibition was accompanied by an oxidation of GSH to GSSG (table 2). In the other systems there was no apparent effect of GSH on formaldehyde formation, and the production of GSSG was very limited. With ethylmorphine, no inhibition of formaldehyde production by GSH was observed in any of the experimental systems.

The HRP/H_2O_2-catalyzed metabolism of aminopyrine was associated with oxygen uptake (table 3). This oxygen uptake was stimulated several-fold by the addition of GSH. A similar stimulated oxygen uptake, in the presence of GSH, was observed with dimethylaniline but not with ethylmorphine.

4. DISCUSSION

The formation of substrate radicals of aminopyrine and dimethylaniline during peroxidase-catalyzed *N*-demethylation has previously been shown by ESR spectroscopy [6,8,16] and is further supported by this finding that the formation of formaldehyde from both drugs in the HRP/H_2O_2 system is markedly inhibited by GSH which undergoes concomitant oxidation to GSSG (cf. tables 1,2). Since GSSG is not formed in the absence of drug substrate, this observation implies that GSH reacts with a drug radical intermediate to form a thiyl radical which can subsequently dimerize to yield GSSG. Consequently, in presence of GSH the radical intermediate is reduced back to the parent drug, and *N*-demethylation is inhibited. It is of interest to note that ethylmorphine, which is rapidly demethylated by liver microsomes in the presence of NADPH, cannot serve as a substrate in the peroxidase-mediated reaction. This is probably due to steric hindrance at the nitrogen atom prohibiting hydrogen abstraction.

The radical intermediates formed from aminopyrine and dimethylaniline react rather poorly with O_2. In contrast, the thiyl radical produced by the interaction of GSH with these intermediates appears to react readily with molecular oxygen (cf. table 3). The nature of any oxygen containing product(s) of GSH is presently unknown but in analogy with what has been observed with other thiyl radicals [17], a GSH sulfonic acid may be one of the products. The identity of the oxygenated GSH product(s), and the physiologial significance of their formation, are presently under investigation in our laboratory.

When the HRP/H_2O_2 system was replaced by liver microsomes + cumene hydroperoxide or NADPH, there was no apparent inhibition of formaldehyde production from either drug by GSH, nor was there any significant stimulation of GSSG formation in the presence of drug (cf. tables 1,2). Thus, it appears that *N*-demethylation of aminopyrine, dimethylaniline and ethylmorphine in liver microsomes does not involve the formation of radical intermediates or, if they are formed, they are not able to react with GSH. Although formation of a radical intermediate of aminopyrine by cytochrome P450 in the presence of cumene hydroperoxide has been reported [8], this species was only observed at >20 mM drug which is far more than used here.

These results indicate that the consumption of GSH observed during cytochrome P450-linked *N*-demethylation of ethylmorphine and other drugs in isolated hepatocytes cannot be due to non-enzymatic oxidation of GSH by radical intermediates, since such radicals do not appear to be formed by cytochrome P450. We are therefore presently investigating whether formation and excretion of unstable conjugates of GSH and product(s) of the *N*-demethylation reaction may be responsible for the depletion of GSH observed in the intact cell system.

ACKNOWLEDGEMENT

This study was supported by a grant from the Swedish Medical Research Council (proj. no.03X-2471).

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