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HYDROPEROXIDE-DEPENDENT ACTIVATION OF *p*-PHENETIDINE CATALYZED BY PROSTAGLANDIN SYNTHASE AND OTHER PEROXIDASES

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Abstract—*p*-Phenetidine is metabolized by ram seminal vesicle (RSV) microsomes, horseradish peroxidase (HRP) and rat liver microsomes to protein-binding products. These reactions are very rapid and depend on the presence of arachidonic acid (AA) or various hydroperoxidases. The RSV- and HRP-mediated binding was inhibited more than 80% by the addition of reduced glutathione (1 mM) or the antioxidant butylated hydroxyanisole (0.5 mM). Indomethacin (100 μM) and acetylsalicylic acid (1 mM) reduced the AA-dependent reaction in RSV microsomes to less than 5% of control values. When hydrogen peroxide replaced AA, the RSV/H_2O_2-supported binding in the presence of 50 μM *p*-phenetidine proceeded at rates similar to that observed with RSV/AA. Unlike the AA-dependent reaction, the H_2O_2-supported reaction showed no inhibition of protein binding at higher *p*-phenetidine concns. The data in this report are consistent with a peroxidatic activation of *p*-phenetidine possibly involving an amine radical catalyzed by prostaglandin synthase (PGS) present in RSV microsomes as well as by other peroxidases. The mechanism for this activation and physiological implications are discussed.

The analgesic and antipyretic substance phenacetin has been found to exert different toxic effects both in man and experimental animals, especially on the kidney and lower urinary tract, including renal necrosis [1, 2] and tumours [3, 4]. One of its primary metabolites, paracetamol, was reported to be involved in the development of the necrotic lesion [5]. In contrast to the hepatotoxicity of paracetamol which is believed to be catalyzed by cytochrome P-450, the renal damage has been suggested to be mediated by prostaglandin synthase (PGS). This enzyme is present in relatively high concns in the kidney medulla, and was demonstrated to catalyze the activation of paracetamol to a reactive intermediate capable of binding to tissue proteins as well as forming a conjugate with glutathione [6–9]. In these reactions, arachidonic acid (AA) is required for activation and indomethacin, an inhibitor of prostaglandin cyclooxygenase, prevents the formation of the reactive metabolite. Furthermore, organic and lipid hydroperoxidases can also support the PGS-dependent activation of paracetamol [6, 8], suggesting that the peroxidase function of the enzyme is involved in the activation process.

In a recent study, where we investigated the role of PGS in the metabolism of several phenacetin metabolites, we found that the deacetylated metabolite *p*-phenetidine induced DNA strand breaks in cultured human skin fibroblasts when they were coincubated with ram seminal vesicle (RSV) microsomes and AA [10]. In contrast to this observation, the PGS-catalyzed activation of paracetamol did not result in formation of such a product. In the present study we therefore examined the peroxidatic oxidation of *p*-phenetidine as well as the substrate requirement in order to further characterize this metabolic activation step.

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MATERIALS AND METHODS

Materials

AA, linolenic acid, soybean lipoxygenase, reduced glutathione (GSH), butylated hydroxyanisole (BHA), indomethacin and horseradish peroxidase (HRP) (Type VI) (250 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). [2,6-^{14}C]*p*-Nitrophenol was obtained from ICN, CA.

Preparation of [^{14}C]p-*phenetidine.* Fifty milligrams [2,6-^{14}C]*p*-nitrophenol (~ 50 μCi, 0.36 mmoles) and 112 mg ethyl iodide (0.72 mmoles) were dissolved in 5 ml dry acetone and refluxed gently for 16 hr in the presence of 140 mg of anhydrous potassium carbonate (1 mmole). The potassium carbonate was filtered off and the acetone evaporated to dryness under nitrogen. Ten millilitres of water was added and the mixture was extracted with benzene (2 × 10 ml). The combined benzene layers were washed successively with 10% sodium hydroxide solution (1 × 10 ml) and water (3 × 10 ml), dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 10 ml of ethanol and catalytically hydrogenated at room temp and pressure in the presence of ~ 100 mg of 5% palladium on charcoal. At the end of 4 hr the catalyst was filtered off and the ethanol evaporated under nitrogen to yield a brownish residue of *p*-phenetidine. The hydrochloride was prepared by dissolving the residue in ~ 150 μl of acetone and adding 60 μl of conc. HCl. The pre-

cipitate was collected by centrifugation and washed once with ice-cold acetone followed by ice-cold ether. The yield of *p*-phenetidine hydrochloride was ~ 25 mg.

Linolenic acid hydroperoxide and 15-hydroperoxy AA were synthesized according to Funk *et al.* [11]. RSV were obtained from a local slaughterhouse and stored at −70° until used. Other chemicals were of analytical grade and purchased from local commercial suppliers.

Methods

RSV and rat liver microsomes were prepared as described by Egan *et al.* [12] and Ernster *et al* [13], respectively. Protein was determined by the method of Lowry *et al.* [14]. Standard incubations with RSV microsomes contained 1 mg of microsomal protein, 50 μM [^{14}C]*p*-phenetidine hydrochloride (~ 280 cpm/nmole), 1 mM EDTA and 100 mM phosphate buffer, pH 8, in a total vol. of 1 ml. Inhibitors, when included, were dissolved either in water or DMSO and added to the reaction mixture in a vol. of 10 μl or less. Reactions were initiated with 100 μM AA or hydroperoxidases (final concn) and terminated after 30 sec of incubation at 25° by addition of 1 vol. of 20% trichloroacetic acid. Variations from these conditions are given in the legends to the figures. The precipitated protein was washed as described by Jollow *et al.* [15]. For solubilization 1 ml of 2 M NaOH was added and the protein was heated for 1 hr at 55°. After neutralization with hydrochloric acid, aliquots were withdrawn for liquid scintillation and protein determination.

Incubations with HRP/H_2O_2 as the activating system contained 0.2 μg of HRP (0.05 units), 50 μM [^{14}C]*p*-phenetidine hydrochloride, 0.9 mg of heat-denaturated rat liver microsomes, 1 mM EDTA and 100 mM phosphate buffer, pH 8, in a total vol. of 1 ml. Reactions were initiated with 1 mM H_2O_2 (final concn) and carried out for 1 min at 25°. Alterations are given in the legends to the figures. The workup procedure was the same as described earlier. Data in this report are presented as means and ranges of at least two separate experiments.

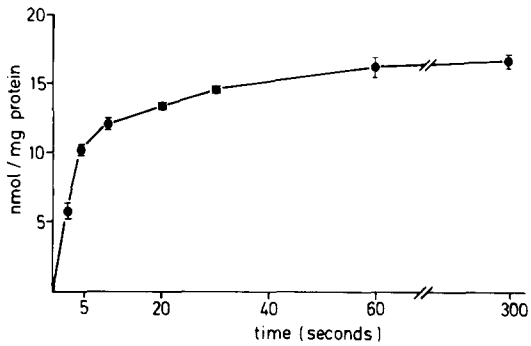

Fig 1. AA-dependent irreversible binding of [^{14}C]*p*-phenetidine to RSV microsomes. Incubations were performed in a total vol. of 6 ml as described in Materials and Methods.

RESULTS

RSV microsomes in the presence of AA catalyzed the rapid irreversible binding of [^{14}C]*p*-phenetidine to proteins (Fig. 1). This reaction was linear for only 5 sec and then gradually levelled off reaching maximal levels after 1 min of incubation. In the absence of AA or when boiled RSV microsomes were used no binding could be detected (Table 1). Incubations under an N_2-atmosphere reduced the binding reaction by 95% as did indomethacin, acetylsalicylic acid, BHA and GSH (Table 1). The AA dependence for the irreversible binding of *p*-phenetidine is shown in Fig. 2. Addition of only 10 μM AA resulted in significant binding whereas optimal rates were obtained at an AA concn of 100 μM. As illustrated in Fig. 3, *p*-phenetidine had a very high affinity for the PGS-catalyzed reaction, a half-maximal reaction rate being observed at 25 μM *p*-phenetidine. At higher substrate concns, however, there was marked inhibition of the binding reaction (Fig. 3).

In order to more specifically study the role of the peroxidase function of PGS in the formation of protein-binding metabolite(s) of *p*-phenetidine, AA was replaced by different hydroperoxidases. Both

Table 1. Irreversible binding of [^{14}C]*p*-phenetidine to RSV microsomes

Incubation conditions	*p*-Phenetidine bound (nmoles/mg protein/30 sec)
Complete system*	15.19 ± 1.24
+ N_2-atmosphere†	0.72 ± 0.27
Boiled microsomes	0
AA	0
+ indomethacin (100 μM)	0.46 ± 0.24
+ acetylsalicylic acid (100 μM)	11.22 ± 0.57
+ acetylsalicyclic acid (1 mM)	0.37 ± 0.14
+ BHA (50 μM)	5.17 ± 0.34
+ BHA (0.5 mM)	0.01 ± 0.01
+ ascorbate (1 mM)	1.88 ± 0.18
+ GSH (100 μM)	6.40 ± 1.40
+ GSH (1 mM)	2.91 ± 0.56

* Incubations were performed as described in Materials and Methods. Values represent means ± S.E. of seven separate experiments.

† Complete incubations were subjected to an N_2-atmosphere 5 min before the addition of AA.

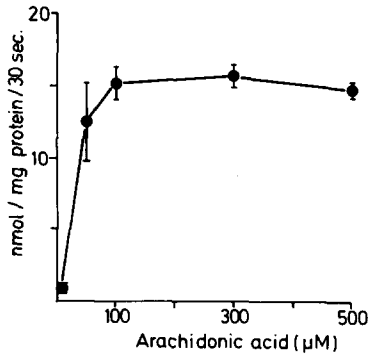

Fig. 2. Effect of AA concn on the irreversible binding of [^{14}C]*p*-phenetidine to RSV microsomes. Incubations were performed as described in Materials and Methods.

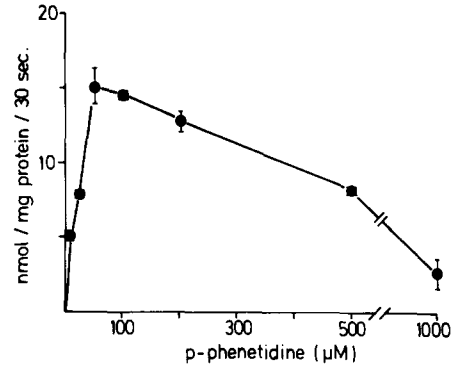

Fig. 3. Effect of *p*-phenetidine concn on the irreversible binding to RSV microsomes. Incubations were performed as described in Materials and Methods.

linolenic acid hydroperoxide and H_2O_2 were found to catalyze the irreversible binding of *p*-phenetidine (Table 2) at rates very similar to AA; 15-hydroperoxy AA was less efficient. Indomethacin did not have any significant effect on these reactions (Table 2). Since H_2O_2 was a good donor substrate for the peroxidase component of PGS, we also investigated its concn dependence for the irreversible binding of *p*-phenetidine to RSV microsomes. As shown in Fig. 4 the reaction was approximately linear up to 100 μM H_2O_2 and was saturated at 1 mM. Higher concns were found to be inhibitory (data not shown). In contrast to the AA-dependent reaction which is inhibited by *p*-phenetidine concns > 50 μM (Fig. 3), the H_2O_2-catalyzed reaction shows increased binding up to a *p*-phenetidine concn of 1 mM (Fig. 5). The H_2O_2-dependent binding with 1 mM *p*-phenetidine is almost 3 times that obtained with AA in the presence of 50 μM *p*-phenetidine after 5 sec of incubation. This demonstrates a higher capacity of PGS to catalyze the activation of *p*-phenetidine utilizing H_2O_2 as a substrate in place of AA.

To further investigate the peroxidatic activation of *p*-phenetidine, HRP/H_2O_2 was used as a model-activating system. As shown in Fig. 6 there was a time-dependent increase in the irreversible binding of 50 and 500 μM *p*-phenetidine when denaturated rat liver microsomes were used as the acceptor protein. These reactions were linear for approximately 5 min of incubation and the rate was almost 3 times higher at 500 μM *p*-phenetidine. Oxygen is not required for the reaction but the absence of either HRP or H_2O_2 decreased the binding reaction by 70 and 90%, respectively (Table 3). The addition of GSH or BHA to the incubations inhibited the binding by 85 and 91%, respectively (Table 3). Of great importance for the HRP/H_2O_2 activation is the amount of enzyme used in the experiments. This is demonstrated in Fig. 7A, displaying a linear increase in the irreversible binding up to 1 μg HRP/ml. The concn of H_2O_2 in these experiments was 1 mM, which was found to be saturating under the incubation condition used (Fig. 7B). Examination of the effect of *p*-phenetidine concn on the HRP/H_2O_2-catalyzed reaction showed increased rates of binding at all concns studied up to 1 mM *p*-phenetidine (Fig. 8). The half-maximal reaction rate was achieved at 100 μM *p*-phenetidine.

In previous studies [16, 17], it was demonstrated that cytochrome P-450 can function as a peroxidase in the presence of various organic hydroperoxides, especially cumene hydroperoxide. We therefore examined if this enzyme also could metabolize *p*-phenetidine to protein-binding products in the presence of cumene hydroperoxide. For comparison experiments using NADPH as cofactor were also performed. As seen in Fig. 9, cumene hydroperoxide catalyzed the irreversible binding of *p*-phenetidine to rat liver microsomes at a rate approximately 12 times that of the NADPH-dependent reaction after 10 min of incubation. *p*-Phenetidine showed some degree of nonspecific binding but this was subtracted from the data in Fig. 9.

DISCUSSION

The results in this report show that PGS in RSV microsomes catalyzes the metabolic activation of

Table 2. Hydroperoxide-dependent irreversible binding of [^{14}C]*p*-phenetidine to RSV microsomes

Addition	*p*-Phenetidine bound (nmoles/mg protein/30 sec)
Linolenic acid hydroperoxide (100 μM)	13.30 ± 0.89
+ indomethacin (100 μM)	11.46 ± 0.35
15-Hydroperoxy AA (100 μM)	5.50 ± 0.10
+ indomethacin (100 μM)	5.85 ± 0.43
H_2O_2 (1 mM)	16.58 ± 0.19
+ indomethacin (100 μM)	14.83 ± 1.08

Incubations were performed as described in Materials and Methods.

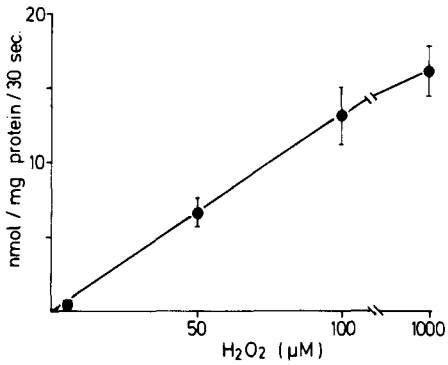

Fig. 4. Effect of H_2O_2 concn on the irreversible binding of [^{14}C]*p*-phenetidine to RSV microsomes. Incubations were performed as described in Materials and Methods.

p-phenetidine to product(s) that bind irreversibly to microsomal protein. This reaction, which is very rapid, occurs during the metabolism of AA and seems to be a result of the peroxidase function of PGS. This conclusion is supported by data in Tables 1 and 2, showing inhibition of the irreversible binding when incubations were performed in the absence of AA or in the presence of added indomethacin as well as by hydroperoxide-mediated binding. Furthermore, HRP/H_2O_2 is also shown to catalyze the binding of *p*-phenetidine to microsomal protein in a reaction which parallels the PGS/AA-dependent reaction, thus providing additional evidence for a peroxidatic activation of this compound.

p-Phenetidine appears to have very high affinity for the PGS/AA-mediated reaction, since substantial binding was observed at only 10 μM substrate concn (Fig. 3). Maximal binding rate was observed at 50 μM *p*-phenetidine and was more than 5 times that obtained with 200 μM paracetamol under similar conditions [8]. However, higher concns of *p*-phenetidine were shown to be inhibitory, possibly due to a direct action on the cyclooxygenase component of PGS, thus decreasing the supply of hydroperoxide to the reaction. When HRP/H_2O_2 was used as the activating system, no such inhibition was observed (Fig. 8). Instead, common Michaelis–Menten kinetics were obtained, indicating that *p*-phenetidine does not inhibit the peroxidase activity but, rather, saturates the enzyme.

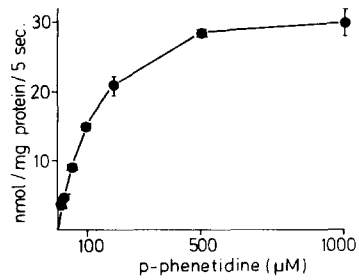

Fig. 5. Effect of *p*-phenetidine concn on the H_2O_2-dependent irreversible binding to RSV microsomes. Incubations were performed for 5 sec in the presence of 1 mM H_2O_2 as described in Materials and Methods.

Of particular interest is the PGS-dependent activation of *p*-phenetidine in the presence of H_2O_2. It is generally believed that the peroxidase activity of PGS has a requirement for a lipid or organic hydroperoxide, and Marnett and Reed [18] have shown that formation of quinones from benzo[*a*]pyrene is much less when H_2O_2 is used as substrate in the place of AA or 15-hydroperoxy AA. However, our work demonstrates that H_2O_2 is equal to AA in supporting the irreversible binding of *p*-phenetidine to RSV microsomes. It is evident that H_2O_2 is a substrate for the peroxidase activity of PGS, since indomethacin, a well-known inhibitor of the cyclooxygenase component, had no effect on *p*-phenetidine binding. In addition there was no inhibition of the binding reaction up to 1 mM *p*-phenetidine concn (Fig. 5), which is similar to the HRP/H_2O_2 system. Even at 5 μM H_2O_2 there was a significant binding of *p*-phenetidine and this is a concn close to that which can be generated by leucocytes in glomerular capillaries [19]. Furthermore it has been reported that H_2O_2 stimulates phospholipase activity in isolated glomeruli and thus increases the availability of AA [19]. It therefore seems possible that H_2O_2 may be of physiological importance *in vivo*, either directly by serving as a substrate for the peroxidase activity of PGS or indirectly by increasing the amount of AA which then can be metabolized by PGS, thereby supporting the peroxidatic activation of *p*-phenetidine.

Recently we have shown that *p*-phenetidine induces DNA strand breaks in human skin fibroblasts [10], which offers an explanation for the genotoxic effect produced by phenacetin. In the present study, *p*-phenetidine is shown to be metabolized to product(s) capable of binding to tissue proteins. This suggests that *p*-phenetidine also might contribute to acute toxic effects of phenacetin, which may be mediated by a peroxidatic activation of the compound both in the liver by cytochrome P-450 and in the kidney by PGS.

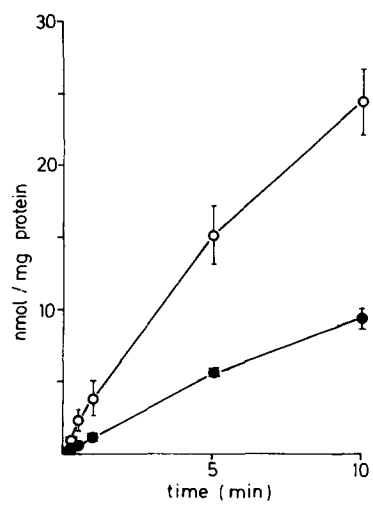

Fig. 6. HRP/H_2O_2-mediated irreversible binding of [^{14}C]*p*-phenetidine to boiled rat liver microsomes. Incubations were performed with 50 μM (●) and 500 μM (○) *p*-phenetidine in a total vol. of 6 ml as described in Materials and Methods.

Table 3. HRP/H_2O_2-catalyzed irreversible binding of [^{14}C]*p*-phenetidine to rat liver microsomes

Incubation conditions	nmoles *p*-phenetidine bound/mg protein/min
Complete system*	1.61 ± 0.24
+ N_2-atmosphere†	1.21 ± 0.11
− HRP	0.48 ± 0.12
− H_2O_2	0.17 ± 0.01
+ GSH (1 mM)	0.25 ± 0.05
+ BHA (0.5 mM)	0.15 ± 0.02

* Incubations were performed as described in Materials and Methods. Values represent means ± S.E. of four different experiments.

† Complete incubations were subjected to N_2-atmosphere 5 min before the addition of H_2O_2.

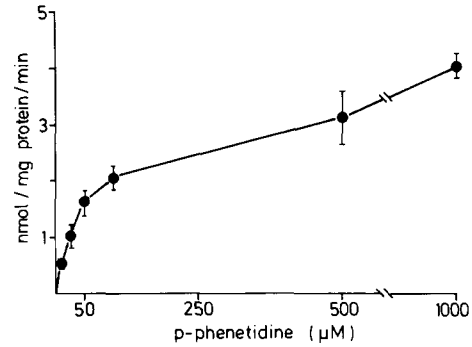

Fig. 8. Effect of *p*-phenetidine concn on the HRP/H_2O_2-mediated irreversible binding to rat liver microsomes. Incubations were performed as described in Materials and Methods.

The nature of the reactive metabolite(s) of *p*-phenetidine as well as its formation is at present unclear. However, preliminary results on peroxidase-mediated metabolism indicate formation of various dimers and trimers of *p*-phenetidine, which suggests an initial formation of an amine radical, possibly resulting from cooxidation in the PGS-catalyzed reaction. The inhibition caused by GSH and the antioxidant BHA of both the PGS/AA- and HRP/H_2O_2-mediated binding (Tables 1 and 3) adds further support to the existence of such a radical. The exact mechanism of the GSH-dependent inhibition is not completely understood. We previously reported on rapid oxidation of GSH during the reaction [10], and are now also looking for the possible formation of a GSH conjugate. Since the half-life of the amine radical might be very short, it seems more likely that some condensation or further oxidation product might be the binding species. Whether or not these metabolites are also involved in the interaction with DNA is presently under investigation in our laboratory.

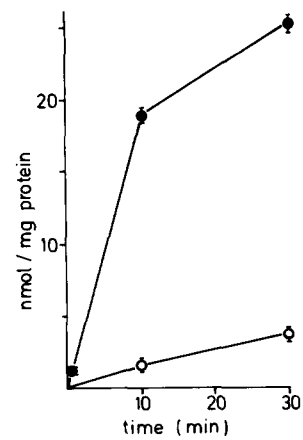

Fig. 9. Cytochrome P-450 catalyzed irreversible binding of [^{14}C]*p*-phenetidine to liver microsomes from phenobarbital-treated rats in the presence of 1 mM cumene hydroperoxide (●) or an NADPH-generating system (○). Incubations were performed at 37° in 0.1 M phosphate buffer, pH 7.4, containing 1 mg/ml microsomal protein; 0.5 mM [^{14}C]*p*-phenetidine hydrochloride and 1 mM EDTA.

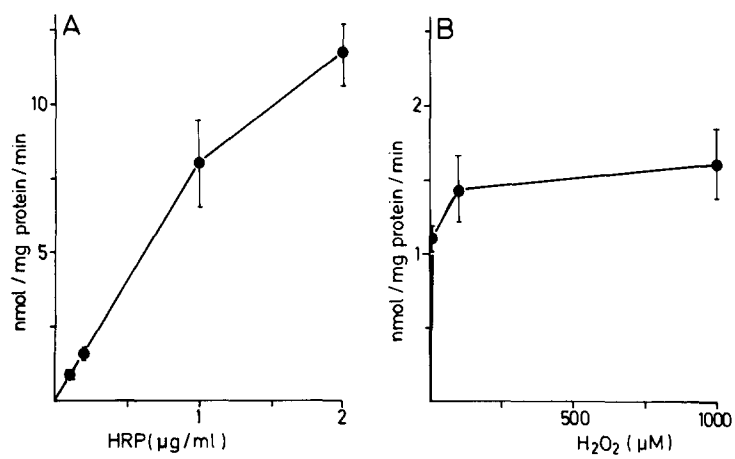

Fig. 7. Effect of HRP (A) and H_2O_2 (B) concns on the HRP/H_2O_2-mediated irreversible binding of [^{14}C]*p*-phenetidine to rat liver microsomes. Incubations were performed as described in Materials and Methods.

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