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PROSTAGLANDIN SYNTHETASE CATALYZED ACTIVATION OF PARACETAMOL

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Abstract—Prostaglandin synthetase has the ability to catalyze the metabolism of paracetamol to a reactive metabolite, which binds to protein and reduced glutathione (GSH). This was demonstrated with microsomes isolated from both sheep seminal vesicles (SSV) and rabbit kidney medulla. The activation of paracetamol occurred through cooxygenation during prostaglandin biosynthesis, the peroxidase activity of this enzyme being responsible for the reaction. In addition to being metabolized, paracetamol also stimulated the rate of prostaglandin biosynthesis, probably by serving as a potent hydrogen donor. The metabolism of paracetamol to a reactive metabolite most likely involved the formation of a paracetamol radical species. This was indicated by an inhibitory effect of the antioxidant, butylated hydroxyanisole, as well as by a very rapid oxidation of GSH during the course of the prostaglandin synthetase catalyzed reaction. Whether this paracetamol radical is further oxidized to the quinone imine prior to reacting with GSH or protein, remains to be established. The ultimate reactive metabolite is evidently the same as that formed with liver microsomes and NADPH since the glutathione conjugates were apparently identical. The rate of paracetamol activation by SSV microsomes was, however, more than 100 times that by liver microsomes and furthermore the apparent K_m was considerably lower. Finally, *N*-OH paracetamol was shown to be activated by prostaglandin synthetase in the presence of arachidonic acid to a metabolite apparently different from that formed from paracetamol.

Paracetamol (acetaminophen) is a widely used analgesic and antipyretic drug which is considered to be nontoxic at therapeutic concentrations, but produces liver damage when taken in overdoses [1–3]. It is currently believed that the microsomal cytochrome P-450 linked monooxygenase system is responsible for activating paracetamol in the liver to an electrophilic intermediate that can bind covalently to cellular macromolecules to produce cell damage [3]. In the presence of reduced glutathione (GSH), the reactive species is trapped as the corresponding glutathione conjugate [4, 5].

The mechanism of activation by cytochrome P-450 as well as the nature of the reactive metabolite is still unknown. It has generally been believed that cytochrome P-450 catalyzes the formation of *N*-OH paracetamol which then spontaneously forms the very reactive quinone imine [2]. *N*-Hydroxylation of paracetamol has however recently been proved not to take place [6] even though the paracetamol quinone imine is still believed to be the ultimate reactive metabolite of paracetamol [7, 8].

Paracetamol is also nephrotoxic [1, 9, 10] and has been shown to bind covalently to protein, especially in the renal medulla, as well as cause depletion of renal GHS [10]. However, since the cytochrome P-450 level and thus the activation of paracetamol by this enzyme is low in the kidney, especially in the medulla, the site of the nephrotoxicity, a cytochrome P-450 activation in this organ is an unlikely explanation as a reason for the nephrotoxicity of this drug.

An enzyme which is present in high concentrations in the kidney medulla is prostaglandin synthetase (PGS) [11]. This enzyme has the ability to catalyze the metabolism of several known carcinogens like benzo(a)pyrene [12], benzidine [13] and *N*-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide [14]. This has been demonstrated using microsomes from both sheep seminal vesicles (SSV) and rabbit kidney inner medulla. The PGS catalyzed metabolism occurs through cooxidation and is due to the hydroperoxidase rather than the fatty acid cyclooxygenase activity of the enzyme [12–14].

We have recently shown that paracetamol may also undergo a PGS catalyzed reaction to a product which is able to form a conjugate with GSH [15]. The reaction was observed in SSV microsomes, was rapid and had high affinity for paracetamol.

The aim of the present study is to further characterize this type of activation in SSV and its activity in the rabbit kidney medulla. The mechanism of activation of paracetamol, as well as the possible *in vivo* implications for this type of activation, primarily for the nephrotoxicity of paracetamol, is discussed.

MATERIALS AND METHODS

Materials

Arachidonic acid, linolenic acid, soybean lipoxygenase, indomethacin, paracetamol and reduced glutathione were obtained from Sigma Chemical Company, (St. Louis, MO). [^{3}H]Paracetamol (sp. act. 3.7 Ci/mmole, purity > 99%) was obtained from New England Nuclear (Boston, MA). N-OH-Paracetamol was a gift from Dr R. Andrews, Sterling Winthrop (Newcastle-upon-Tyne, U.K.). Linolenic acid hydroperoxide was synthesized according to the method by Funk *et al.* [16]. Frozen (−70°) sheep seminal vesicular glands were kindly supplied by Ms

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Other chemicals were obtained from local commercial suppliers.

Methods

Microsomes from sheep seminal vesicular glands and rabbit kidney medulla were prepared as previously described [14, 17, 18, 21] and the protein content was measured according to the method of Lowry *et al.* [19].

Incubations were performed at 25° in 0.1 M phosphate buffer, pH 8.0, at a protein concentration of 1 mg per ml of incubate. The final concentration of arachidonic acid (AA) was generally 0.3 mM and that of GSH 2.5 mM. Reactions were initiated with AA or linolenic acid hydroperoxide and terminated by the addition of 0.2 ml 3N perchloric acid per ml of incubation mixture. Incubations with kidney microsomes were performed at 37° and at an arachidonic acid concentration of 0.1 mM.

Paracetamol glutathione conjugate formation was determined by high-performance liquid chromatography (HPLC) as described earlier [5]. In experiments where *N*-OH paracetamol was used as substrate the elution media contained 0.01% of Desferal.

Experiments on covalent binding of [^{3}H]-paracetamol to microsomal protein were carried out as described for paracetamol–glutathione conjugate formation but without added GSH. Reactions were terminated by addition of 1 ml of 20% trichloroacetic acid per ml incubate and the covalent binding was determined by the method of Jollow *et al.* [3].

Reduced and oxidized glutathione were determined by HPLC according to the method of Reed and coworkers [20].

Oxygenation of arachidonic acid was monitored with a Clark electrode (Yellow Springs Instrument Co.) in 100 mM phosphate buffer, pH 8.0, at 30°. 1 mg SSV microsomes were incubated with paracetamol in a total volume of 2 ml and reactions were started with 100 μM arachidonic acid. The oxygen consumption rate was calculated from the linear part of the recorder graph after arachidonic acid addition.

RESULTS

In the presence of arachidonic acid, paracetamol and GSH, microsomes isolated from sheep seminal vesicles (SSV) catalyzed the formation of glutathione conjugate of paracetamol (Table 1). This reaction, which has previously been shown to be enzymatic in nature [15] was dependent on oxygen availability and varied with temperature. It was inhibited by indomethacin, a potent inhibitor of prostaglandin synthetase (PGS) and also by the antioxidant butylated hydroxy anisole (BHA). The cytochrome P-450 inhibitor, metyrapone, exhibited no effect on this system (Table 1). These, and previously published data, strongly indicate involvement of PGS in the activation of paracetamol.

As shown in Fig. 1, the activity was also dependent on microsomal protein concentration with an almost linear increase up to 1 mg protein per ml of incubation mixture. The reaction was maximal at an arachidonic acid concentration of about 0.3 mM (Fig. 1B) but a concentration of as high as 1 mM GSH

Table 1. Arachidonic acid dependent paracetamol–glutathione conjugate formation in SSV microsomes

	nmoles/min per mg protein
Control	27.8
+ Indomethacin (100 μM)	5.7
+ Metyrapone (1 mM)	25.9
N_2 atmosphere	5.9
4°	21.5
37°	34.3
BHA (100 μM)	11.8
BHA (500 μM)	N.D.

Incubations were performed for 1 min at 25° as described in Methods. The inhibitors were preincubated with microsomes for 2 min.

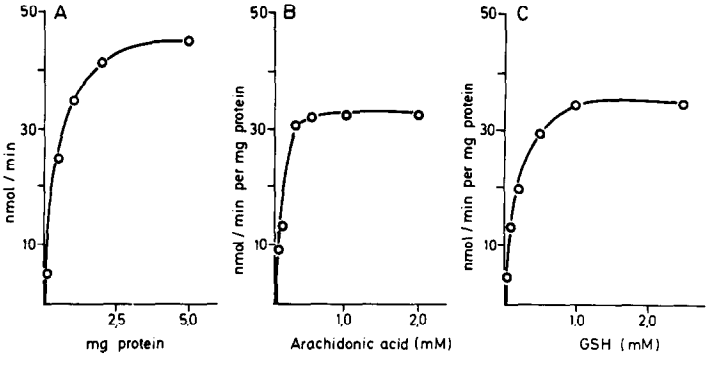

Fig. 1. Effect of protein- (A), AA- (B) and GSH- (C) concentrations on paracetamol glutathione conjugate formation in SSV microsomes. Time of incubation was 1 min.

was needed for the maximal rate of paracetamol conjugate formation (Fig. 1C).

The formation of a paracetamol–glutathione conjugate is a reflection of prior activation of paracetamol to a reactive metabolite which then conjugates with GSH [4, 5]. This conjugation may be nonenzymatic but may also be catalyzed by glutathione transferases (GST). In the reaction described here the conjugation is apparently nonenzymatic, since the SSV microsomes were devoid of any measurable GST activity. There was also no observable increase in paracetamol–glutathione conjugate formation, even at the lowest GSH concentration tested, if liver cytosol containing GST activity was added to the reaction mixture. This implies that the conjugation of the reactive paracetamol metabolite with GSH is preferentially nonenzymatic even at low GSH concentrations. Moreover, the rate limiting step of the paracetamol–glutathione conjugate formation at concentrations of GSH higher than 1 mM is thus the formation of the reactive paracetamol metabolite.

In addition to forming a conjugate with paracetamol, GSH is rapidly oxidized in the presence of AA and paracetamol (Fig. 2). In fact, the rate of oxidation to GSSG was almost five-fold that of the formation of paracetamol–glutathione conjugate. Some GSH oxidation does also occur in the absence of paracetamol probably due to the peroxidase activity of PGS.

Hepatic cytochrome P-450 is also capable of catalyzing the formation of a reactive paracetamol metabolite which conjugates with glutathione. The affinity of cytochrome P-450 for this reaction is, however, very low, as is the rate of the reaction [5].

The affinity and rate of activation of the cytochrome P-450 dependent and the prostaglandin synthetase dependent reactions are presented in Table 2. The rate of formation of paracetamol–glutathione was more than 100-fold higher whilst the K_m was about 10-fold less if SSV microsomes and AA were used rather than mouse liver microsomes and NADPH. However, even though the initial rate of reaction with SSV microsomes was higher, the reaction was linear for less than 1 min whereas the cytochrome P-450 dependent reaction was linear for more than 1 hr.

The identity of the paracetamol–glutathione conjugate formed by the PGS catalyzed reaction appeared to be the same as the one formed from the cytochrome P-450 dependent reaction. This is apparent from the identical retention times (10, 7 min) on HPLC of the two glutathione conjugates.

Table 2. K_m and V_{max} for paracetamol–glutathione conjugate formation in microsomes from SSV and mouse liver

Microsome source	K_m (μM)	V_{max} (nmoles/min per mg protein)
SSV*	54	100
Mouse liver†	429	0.75

* In the presence of arachidonic acid (0.3 mM).
† In the presence of an NADPH generating system.

In order to by-pass the cyclooxygenase step of the PGS in SSV microsomes, AA was replaced by a lipid hydroperoxide, linolenic acid hydroperoxide. This hydroperoxide was able to support the paracetamol–glutathione conjugate formation in the presence of SSV microsomes (Table 3). This activity, which was slightly lower than with AA, was not inhibited by indomethacin or by N_2, but was inhibited by BHA.

The activation of paracetamol by SSV microsomes could also be illustrated using covalent binding to protein if GSH was omitted from the incubation mixture (Fig. 3). The rate of covalent binding was similar to the formation of the glutathione conjugate in that it was initially rapid, but levelled off after about a minute of incubation. The amount of covalently bound paracetamol was, however, much less than the amount of glutathione conjugate formed. The covalent binding was almost completely inhibited in the presence of GSH and, as with the

Table 3. Linolenic acid hydroperoxide dependent paracetamol–glutathione conjugate formation in SSV microsomes

	nmoles/min per mg protein
Control	15.0
Indomethacin (100 μM)	16.3
N_2 atmosphere	14.7
BHA (100 μM)	8.7
BHA (500 μM)	2.8

Incubation was performed for 1 min at 25° as described in Methods. Inhibitors were preincubated with microsomes for 2 min.

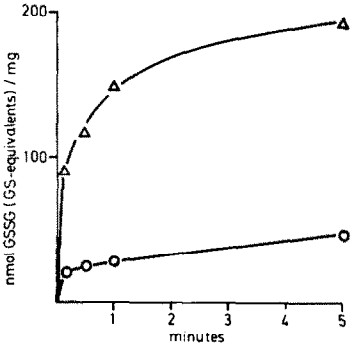

Fig. 2. Oxidation of GSH by SSV microsomes in the presence of AA (0.2 mM) (○) or AA + paracetamol (200 μM) (△). The initial GSH concentration was 1 mM.

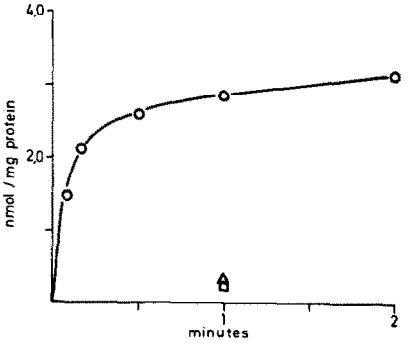

Fig. 3. Covalent binding to protein of paracetamol in SSV microsomes (○). Additions: GSH, 1 mM (□); indomethacin, 0.1 mM (△). Paracetamol concentration was 0.2 mM.

paracetamol–glutathione conjugate formation it was also inhibited by the addition of indomethacin.

Formation of *N*-OH paracetamol has been suggested to be the first step in the formation of the reactive metabolites of paracetamol in liver [2]. *N*-OH paracetamol then spontaneously forms *N*-acetylquinone imine which is believed to be the reactive species of paracetamol. When *N*-OH paracetamol was incubated together with SSV microsomes, AA and GSH, a glutathione conjugate was rapidly formed; a conjugate which had a different retention time (10.9 min) on HPLC compared to that formed from paracetamol. The rate of formation of this conjugate corresponded well to that formed from paracetamol and the activity levelled off after a few seconds (Fig. 4).

In the incubation medium, *N*-OH paracetamol rapidly undergoes degradation to the *N*-acetylquinone imine, which may spontaneously conjugate with GSH. Formation of this conjugate was also observed during incubation with *N*-OH paracetamol. This glutathione conjugate had similar retention time on HPLC to that formed from paracetamol incubated with SSV or mouse liver microsomes.

During the synthesis of the various prostaglandins from AA, oxygen is rapidly consumed, reflecting the cyclooxygenase activity of the PGS. The addition of paracetamol markedly increased the AA stimulated oxygen consumption in SSV microsomes (Table 4). The stimulation was more than two-fold and was maximal at 200 μM paracetamol, the concentration which also gave the greatest rate of activation [15]. This increase indicates an increased cyclooxygenase activity and an increase in prostaglandin synthesis which has also been confirmed by measurement of the formation of prostaglandins $F_2\alpha$ and E_2. These results are also in accordance with previous findings by Egan *et al.* [21] who demonstrated a similar increase in oxygen consumption when phenol was present in the reaction mixture.

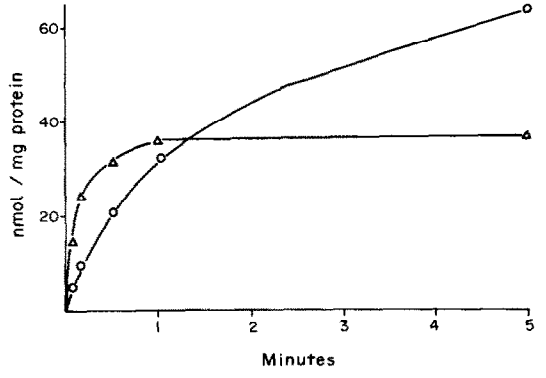

Fig. 4. Formation of glutathione conjugates of *N*-OH paracetamol during incubation with SSV microsomes and AA. (△), represents a glutathione conjugate formed after enzymatic activation of *N*-OH paracetamol. (○), represents the glutathione conjugate formed after spontaneous degradation of *N*-OH paracetamol to the quinone imine. The concentration of *N*-OH paracetamol was 0.2 mM.

Table 4. Effect of paracetamol of the oxygenation of arachidonic acid by SSV microsomes

Additions	Concn (μM)	nmoles O_2/mg protein per min
Arachidonic acid	100	604.8
+ Paracetamol	50	886.2
+ Paracetamol	200	1306.2
+ Paracetamol	1000	1093.2

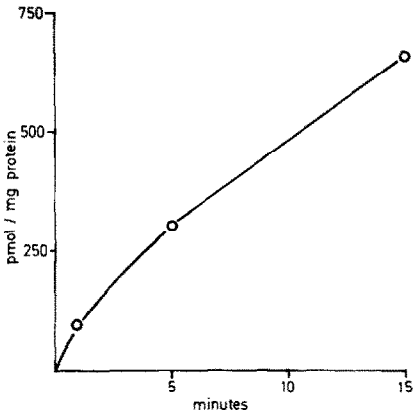

Fig. 5. Arachidonic acid dependent covalent binding to protein of paracetamol in rabbit medullary microsomes. The paracetamol concentration was 0.2 mM.

Activation of paracetamol by rabbit kidney medullary microsomes

Microsomes from rabbit kidney medulla also exhibit a high PGS activity. As with SSV microsomes, rabbit kidney medullary microsomes catalyzed the AA-dependent activation of paracetamol, which resulted in covalent binding of paracetamol to protein (Fig. 5). The rate of covalent binding was, however, much lower, and the reaction was almost linear for 15 min. This reaction was inhibited by indomethacin and not affected by metyrapone (Table 5), as was the case with SSV microsomes. Similar to these results rat kidney microsomes has been demonstrated to catalyze the formation of a paracetamol–glutathione conjugate [22].

DISCUSSION

This and previous studies [15, 22], clearly demonstrate the ability of PGS to catalyze the metabolic activation of paracetamol. This activity is observed with microsomes from both sheep seminal vesicles and rabbit medulla and is demonstrated both as covalent binding to protein and formation of a paracetamol–glutathione conjugate.

Table 5. Covalent binding of paracetamol to protein of rabbit medulla microsomes

Incubation conditions	pmoles/mg per min
Control	42.3
+ Indomethacin, 100 μM	9.1
+ Metyrapone, 1 mM	45.4

Incubations were performed for 10 min at 37° as described in Methods. Inhibitors were preincubated with microsomes for 2 min.

It is evidently the peroxidase activity of PGS, that catalyzes the reduction of the hydroperoxy endoperoxide of arachidonic acid (PGG_2) to the hydroxy-endoperoxide (PGH_2) [23, 24], which is also responsible for the metabolic activation of paracetamol. This conclusion is supported by the observation that AA could be replaced by linolenic acid hydroperoxide which partakes in a reaction not dependent on oxygen or inhibited by indomethacin. This is also in accordance with similar findings with the PGS system using substrates other than paracetamol [12–14].

The mechanism of activation of paracetamol by PGS is not yet established, nor is the nature of the reactive metabolite. Based on the findings described in this and other studies a possible reaction scheme is proposed (Fig. 6). The reaction which is catalyzed by the peroxidase function of PGS probably involves a one electron oxidation reaction which could lead to hydrogen abstraction to yield the phenoxy radical of paracetamol. Support is lent to the formation and existence of such a radical of paracetamol by the inhibitory effect of the antioxidant BHA, and the extremely rapid oxidation of GSH during the course of the reaction. GSH could thus reduce this radical metabolite of paracetamol back to paracetamol. In addition, Nelson and coworkers [25] recently identified a phenoxy radical of paracetamol formed during a reaction catalyzed by horse radish peroxidase. Since this type of reaction is similar to that catalyzed by PGS these results are further support for the assumption that a paracetamol radical intermediate is formed during the PGS reaction. A paracetamol radical may then be further oxidized to the quinone imine prior to reaction with GSH. The radical itself may also react with GSH to form the glutathione conjugate, a reaction which has recently been described [26]. The nature of the GSH conjugating species is presently under investigation.

Clearly *N*-hydroxylation is not involved in the PGS catalyzed activation. In fact, *N*-OH paracetamol could be activated to a reactive metabolite different from that formed from paracetamol as evident from the formation of a glutathione conjugate with a different retention time on HPLC analysis. The activation of *N*-OH paracetamol may thus also involve a hydrogen abstraction. Since the further oxidation of this radical species to a quinone imine is unlikely, this observation favors a direct conjugation of the radical with GSH.

Since the paracetamol glutathione conjugate formed by the PGS-catalyzed reaction is evidently identical to that formed in liver microsomes, it is tempting to speculate on a similar type of activation by cytochrome P-450. The formation of a paracetamol radical species catalyzed by cytochrome P-450 could well be possible. A tentative mechanism for such a reaction has in fact been suggested recently [25, 26].

The *in vivo* relevance as well as the toxicological implications of the PGS catalyzed reaction with paracetamol are presently unclear.

The PGS-catalyzed metabolic activation of paracetamol is of particular interest regarding the nephrotoxicity of the drug. Paracetamol may cause two types of nephrotoxicity. After a massive overdose, in addition to the hepatic lesion, acute tubular necrosis may be produced [1]. This is primarily a lesion of the cortex. With chronic abuse, however, the initial lesion is localized to the medulla. It is this latter type of lesion which could result from a PGS-catalyzed activation of paracetamol. The localization of PGS to the inner medulla as well as the high rate of activation of paracetamol at moderate (therapeutic) doses favours this assumption. As shown in this study, PGS in the medulla of rabbit kidney is indeed able to catalyze the activation of paracetamol (cf. Fig. 5). The rate is, however, quite low. In the kidney medulla paracetamol has also been shown to be a potent inhibitor of the cyclo-

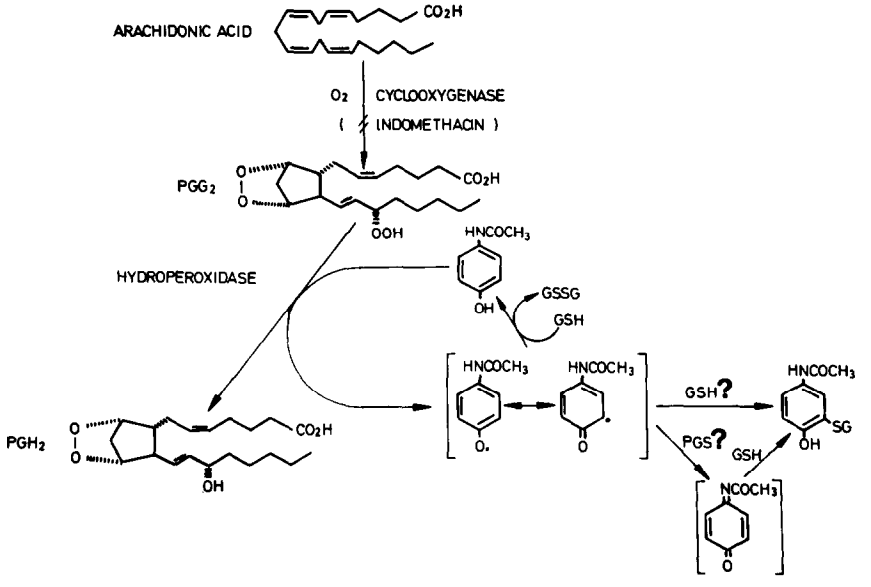

Fig. 6. Possible mechanism for the PGS-catalyzed activation of paracetamol.

oxygenase of PGS [27]. Such inhibition would counteract the peroxidase-catalyzed activation and could, at least partly, explain the low rate of activation.

The inhibitory effect of paracetamol on prostaglandin synthesis in the kidney medulla is opposite to what is found in the sheep seminal vesicles where paracetamol stimulates the synthesis by serving as a potent hydrogen donor. No inhibitory effect on the cyclooxygenase activity could be observed. The reason for this difference, as well as the effect of paracetamol on PGS in other organs, needs to be clarified.

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