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RELATIONSHIP BETWEEN METABOLISM AND CYTOTOXICITY OF *ortho*-PHENYLPHENOL IN ISOLATED RAT HEPATOCYTES

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Abstract—The relationship between the metabolism and the cytotoxicity of *ortho*-phenylphenol (OPP) was investigated using isolated rat hepatocytes. Addition of OPP (0.5–1.0 mM) to the hepatocytes caused a dose-dependent toxicity; 1.0 mM OPP caused acute cell death. Pretreatment of hepatocytes with SKF-525A (50 μM, a non-toxic level) enhanced the cytotoxicity of OPP (0.5–1.0 mM). This was accompanied by inhibition of OPP metabolism. Conversely, OPP at low concentrations (0.5 or 0.75 mM) was converted sequentially to phenyl-hydroquinol (PHQ) and then to glutathione (GSH) conjugate in the cells. The concentrations of both metabolites, especially PHQ–GSH conjugate, were very low in hepatocytes exposed to 1.0 mM OPP alone as well as with SKF-525A. The cytotoxicity induced by 0.5 mM OPP was enhanced by the addition of diethylmaleate (1.25 mM) which continuously depletes cellular GSH. In contrast, additions to hepatocytes of 5 mM of dithiothreitol, cysteine, *N*-acetyl-L-cysteine or ascorbic acid significantly inhibited the cytotoxicity induced by 0.5 mM PHQ; GSH, protein thiols and ATP losses were also prevented. Further, these compounds depressed the rate of PHQ loss in hepatocyte suspensions. These results indicate that the acute cytotoxicity caused by the high dose (1.0 mM) of OPP is associated with direct action by the parent compound; at low doses (0.5–0.75 mM) of OPP, the prolonged depletion of GSH in hepatocytes enhances the cytotoxicity induced by PHQ.

ortho-Phenylphenol (OPP§) and its sodium salt (SOPP) are broad spectrum antimicrobials and are used as a postharvest treatment of fruits and vegetables to prevent microbial decay during transport and storage. Because of their widespread use, the potential toxicities of OPP and SOPP have been investigated extensively both *in vivo* and *in vitro* to assess acute and chronic toxicities [1], cytogenetic effects [2, 3], mutagenicity [4], teratogenicity [5] and immunological effects [6]. In regard to organ toxicity, a single large dose of OPP produced in rats acute hepatic and renal damage [1, 7] and chronic large doses of OPP given to rats caused bladder tumors [8]. Although the exact mechanisms of this hepatic and renal damage, and the induction of bladder tumors by OPP remain unclear, part of the metabolic activation of OPP, quinone formation in particular, has been postulated to play an important role [9, 10]. OPP and SOPP are oxidized by microsomal cytochrome P450 to phenyl-hydroquinol (PHQ, 2,5-dihydroxybiphenyl) once the conjugation processes are saturated [11]. Consequently, oxidation of PHQ, which is a major metabolite, leads to the formation of phenyl-benzoquinone (PBQ) and superoxide anion radicals via PHQ semiquinone [11, 12]. PBQ and these radicals may be responsible for the induction of tumors and organ damage by OPP and SOPP [13, 14]. In previous experiments, we demonstrated that a single large dose of OPP to rats produces acute hepatic damage accompanied by centrilobular necrosis, depletion of tissue glutathione (GSH) and formation of PHQ–GSH conjugates [7]. We demonstrated further that some metabolites derived from OPP were bound covalently to cellular proteins by a microsomal monooxygenase system [9]. Also, addition of OPP or its metabolites (PHQ, PBQ) to isolated rat hepatocytes causes dose-dependent cell death with rapid depletion of ATP, GSH and protein thiols [15]. The toxic effects of PBQ are the most potent, followed by those of OPP and PHQ. To clarify the mechanism of OPP toxicity, and its metabolite, PHQ, the effects of some modulating agents related to the activity of microsomal cytochrome P450 and the level of cellular GSH were studied.

MATERIALS AND METHODS

Materials. The chemical compounds used were obtained from the following companies: OPP (purity > 99%) from the Tokyo Kasei Co. (Tokyo, Japan); PHQ and PBQ (purities > 98%) from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); reduced glutathione (GSH) and its oxidized form (GSSG), bovine serum albumin and collagenase (grade II) from the Sigma Chemical Co. (St Louis, MO, U.S.A.). SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was a gift from

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§ Abbreviations: OPP, *ortho*-phenylphenol; SOPP, sodium OPP; PHQ, phenyl-hydroquinol; PBQ, phenyl-benzoquinone; GSH, reduced glutathione; GSSG, oxidized glutathione; SKF-525A, 2-diethylaminoethyl-2,2-diphenyl-valerate hydrochloride; DEM, diethyl maleate.

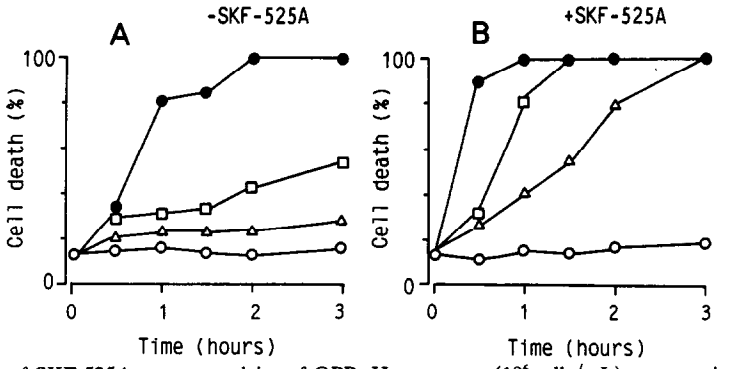

Fig. 1. Effect of SKF-525A on cytotoxicity of OPP. Hepatocytes (10^6 cells/mL) were preincubated in the absence (A) or in the presence (B) of 50 μM SFK-525A for 10 min, and then were incubated with OPP; 0.5 mM (△), 0.75 mM (□), 1.0 mM (●) and 0 mM (○). Results are expressed as the means from three experiments.

Smith, Kline and French Laboratory (Philadelphia, PA, U.S.A.). All other chemicals were of the highest grade of purity commercially available.

Isolation and incubation of hepatocytes. Male Fischer-344 rats (220–280 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of liver as described by Moldéus *et al.* [16]. Hepatocyte viability was assessed using Trypan blue exclusion. Approximately 90% of the freshly isolated hepatocytes routinely exclude Trypan blue.

Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (Hepes). All incubations were performed in a rotating, round-bottom flask at 37° under an atmosphere of 95% O_2 and 5% CO_2. In some experiments, prior to addition of OPP, the isolated hepatocytes were pretreated for 10 min with 50 μM SKF-525A dissolved in water or for 15 min with 1.25 mM diethyl maleate (DEM) dissolved in dimethyl sulfoxide. In the experiments supplemented with sulphydryl compounds, each compound (5 mM) was dissolved in Krebs–Henseleit buffer and was added to the cell suspension 5 min prior to PHQ treatment. Reactions were started by the addition of OPP or PHQ dissolved in dimethyl sulfoxide (final concentration less than 1%). Aliquots of incubation mixture were taken at various times for the determination of cell viability as well as concentration of GSH, GSSG, ATP, protein thiols, protein and OPP and its metabolites.

Biochemical assays. ATP concentrations in hepatocytes were measured using HPLC methodology following the procedure of Jones [17].

Cellular GSH and GSSG levels were determined by HPLC essentially as described by Reed *et al.* [18].

Reduced protein thiols were determined by Ellman's reagent (dithiobis dinitrobenzoic acid) as described previously [19].

Protein was determined by the method of Lowry *et al.* [20] or by the method of Peterson [21] using bovine serum albumin as a standard.

Determination of OPP and its metabolites by HPLC. After aliquots of hepatocyte cell suspensions were treated with a cell disrupter (Sonifier, Brabson Sonic Power Co., Danbury, CT, U.S.A.) in ice water for 20 sec and filtered through membrane cartridges (pore size, 0.45 μm), the eluent was injected onto an analytical TSKgel ODS-120T column (4.6 mm i.d. × 250 mm, Toyo Soda Co., Tokyo) equipped with a UV absorbance detector (254 nm). The mobile phase was methanol–0.1 M ammonium dihydrogen phosphate (50/50, by vol.) and the flow rate was 1.0 mL/min. The recoveries of PHQ and PHQ–GSH conjugates were checked by the determination of known amounts of these authentic compounds added to untreated hepatocyte suspensions at 0°, and both recoveries were greater than 86%. The synthetic PHQ–GSH conjugate was prepared as described previously [9].

RESULTS

Addition of OPP to isolated rat hepatocytes caused cell death accompanied by the depletion of intracellular levels of ATP, GSH and protein thiols [15]. Figure 1 shows that cytotoxicity, which depended on the concentration of OPP, was enhanced by an inhibitor of microsomal monooxygenase, SKF-525A (50 μM); treatment with SKF-525A alone did not affect cell viability during the incubation period. In isolated rat hepatocytes, SKF-525A effectively inhibits not only phase I but also phase II reactions of drug metabolism [22]. As shown in HPLC elution profiles (Fig. 2), the levels of metabolites derived from 0.5 mM OPP in hepatocytes pretreated with the inhibitor were extremely low compared with those from OPP alone. More than 80% of the parent compound remained unmetabolized in the cell suspension pretreated with SKF-525A. Since the amount of PHQ produced in SKF-525A-treated cells was approximately 10% (6 nmol/10^6 cells) of that produced in untreated cells, it is apparent that a concentration of 50 μM SKF-525A was sufficient to inhibit the activity of microsomal cytochrome P450 in hepatocytes. Further, OPP at concentrations of

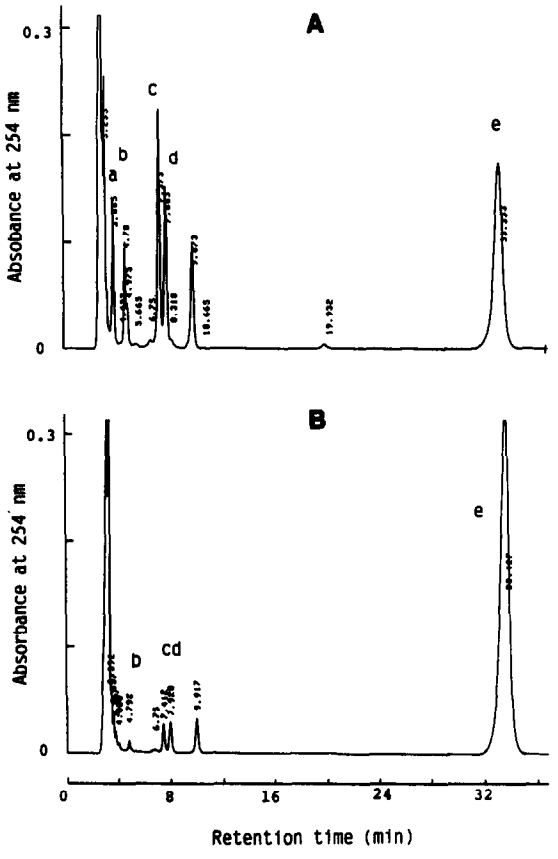

Fig. 2. HPLC elution profiles of metabolites derived from 0.5 mM OPP in hepatocytes. Hepatocytes (10^6 cells/mL) in the absence (A) or presence (B) of 50 μM SKF-525A were incubated with 0.5 mM OPP for 60 min. Peak a; OPP-glucuronide, peak b; PHQ–GSH, peak c; PHQ-glucuronide, peak d; PHQ and peak e; OPP.

0.75 or 1.0 mM was not significantly metabolized in SKF-525A-pretreated hepatocytes after a 3-hr incubation (data not shown). These results indicate that OPP itself is very toxic to hepatocytes when microsomal cytochrome P450 is inhibited by SKF-525A and high concentrations of OPP remain in the cells.

Figure 3 shows the time-course for PHQ and PHQ–GSH conjugate levels in unpretreated hepatocytes incubated with OPP (0.5–0.75 mM). OPP, at a concentration of 0.5 or 0.75 mM, was converted progressively to PHQ or to the PHQ–GSH conjugate with time. In hepatocytes treated with 1.0 mM OPP, however, the concentrations of both metabolites, in particular the PHQ–GSH conjugate, were very low. The levels of PHQ–GSH conjugate in 0.5 mM OPP-treated cells increased with time and were nearly twice those in 0.75 mM OPP-treated cells. On the other hand, the levels of PHQ decreased with time after maximum concentration; this decrease indicates the conversion of PHQ to other metabolites, i.e. PBQ or glucuronide conjugates.

The PHQ produced is converted to the corresponding PBQ via reactive semiquinone radicals [11]. The quinone and semiquinone radicals are reactive intermediates which react with sulfhydryl compounds, protein thiols and other nucleophilic macromolecules [23, 24]. Therefore, we investigated the effects of GSH depletion with DEM at a low toxic level of OPP (0.5 mM) (Fig. 4). After 15 min preincubation with 1.25 mM DEM, cellular GSH levels were depleted to approximately 5 from 55 nmol/10^6 cells (Fig. 4A and B), but increased gradually after 1 hr. Addition of OPP caused rapid GSH loss in unpretreated cells and resulted in complete depletion of GSH in DEM-pretreated cells. When cellular GSH levels were depleted continuously with DEM, the percentage of cell death induced with 0.5 mM OPP increased with time (Fig. 4C and D). Although the pretreatment with DEM did not affect the formation of PHQ (Fig. 4E), the

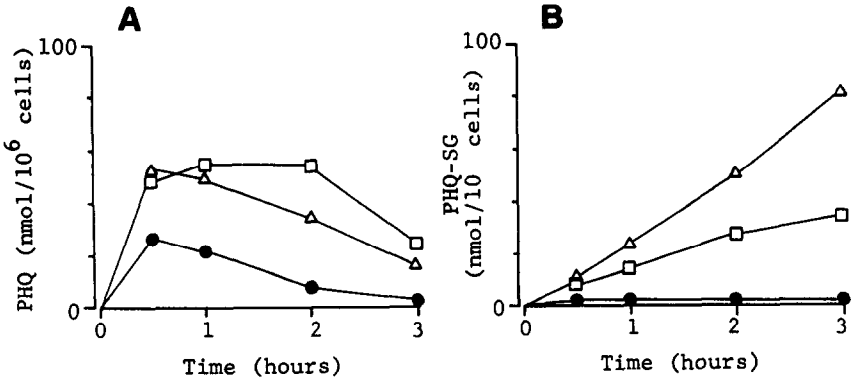

Fig. 3. Changes in the levels of PHQ (A) and PHQ–GSH conjugate (B) in suspensions of hepatocytes treated with OPP; 0.5 mM (△), 0.75 mM (□) and 1.0 mM (●). Untreated hepatocytes (10^6 cells/mL) were incubated with OPP and the amounts of both metabolites were measured as described in Materials and Methods. Results are expressed as the means from three experiments.

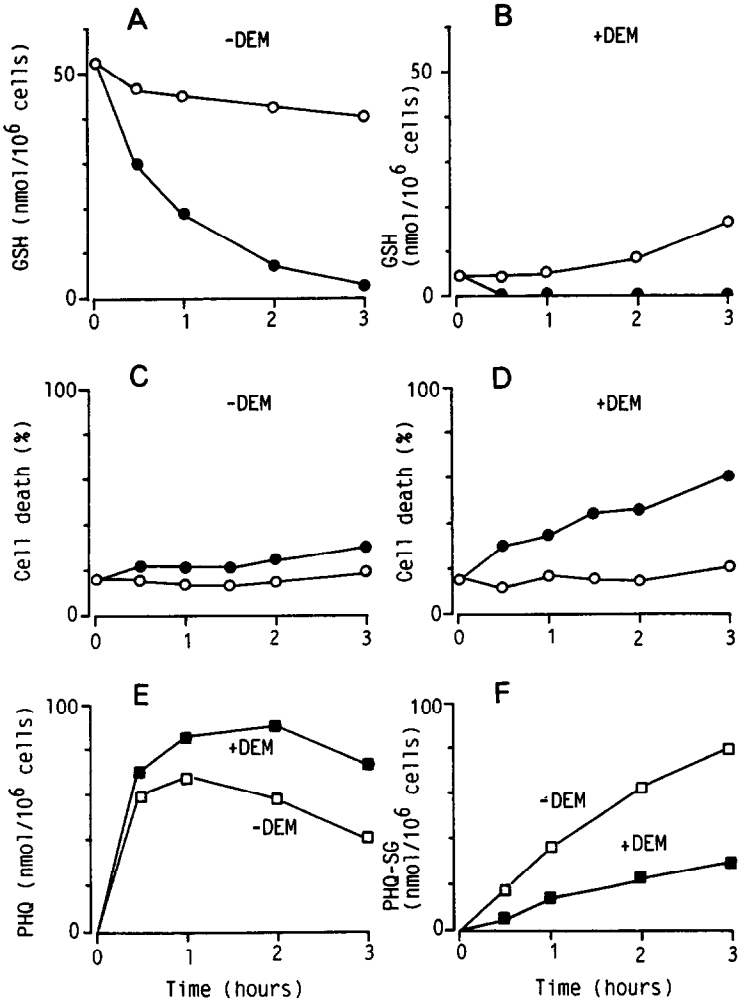

Fig. 4. Effects of DEM on GSH level (A,B), cytotoxicity (C,D), PHQ (E) and PHQ–GSH conjugate (F) on hepatocytes treated with 0.5 mM OPP. Hepatocytes (10^6 cells/mL) were pretreated with or without 1.25 mM DEM for 15 min and then were incubated with no addition (○) or 0.5 mM OPP (●). The amounts of both metabolites derived from OPP in untreated (□) or DEM-treated (■) hepatocytes were determined as described in Materials and Methods. Results are expressed as the means from three experiments.

levels of PHQ–GSH conjugate produced in untreated cells were about three times those in DEM-pretreated cells (Fig. 4F). These results indicate that severe depletion of cellular GSH levels leads to the enhancement of cytotoxicity at low concentrations of OPP.

Because protein thiols and cellular sulphydryl groups may be important targets for reactive intermediates derived from OPP [15], the effects of sulphydryl compounds or ascorbic acid on PHQ-induced cytotoxicity were investigated (Fig. 5). The percentage of cell death induced by PHQ (0.5 mM) was significantly reduced by dithiothreitol, cysteine, or *N*-acetyl-L-cysteine (Fig. 5A). These compounds (5 mM) reduced the rapid loss of cellular ATP, GSH and protein thiols accompanied by the simultaneous inhibition of PHQ loss (Fig. 5B–E). PHQ did not react directly with ATP in the absence of hepatocytes and the loss of ATP from hepatocytes treated with PHQ was accompanied by an increase in cellular levels of ADP and AMP (data not shown). On the other hand, ascorbic acid (5 mM) delayed, but did not prevent, the onset of cell death and the loss of these cellular components following PHQ treatment. Thus, the protective effect of ascorbic acid on PHQ-induced toxicity may be different from that of sulfhydryl compounds. These results indicate that there is a correlation between the onset of cytotoxicity and the loss of PHQ from the hepatocytes.

DISCUSSION

The results of this study indicate that there are at least two mechanisms involved in OPP-induced cytotoxicity. First, OPP itself is a potent toxic compound to hepatocytes and its effect is enhanced

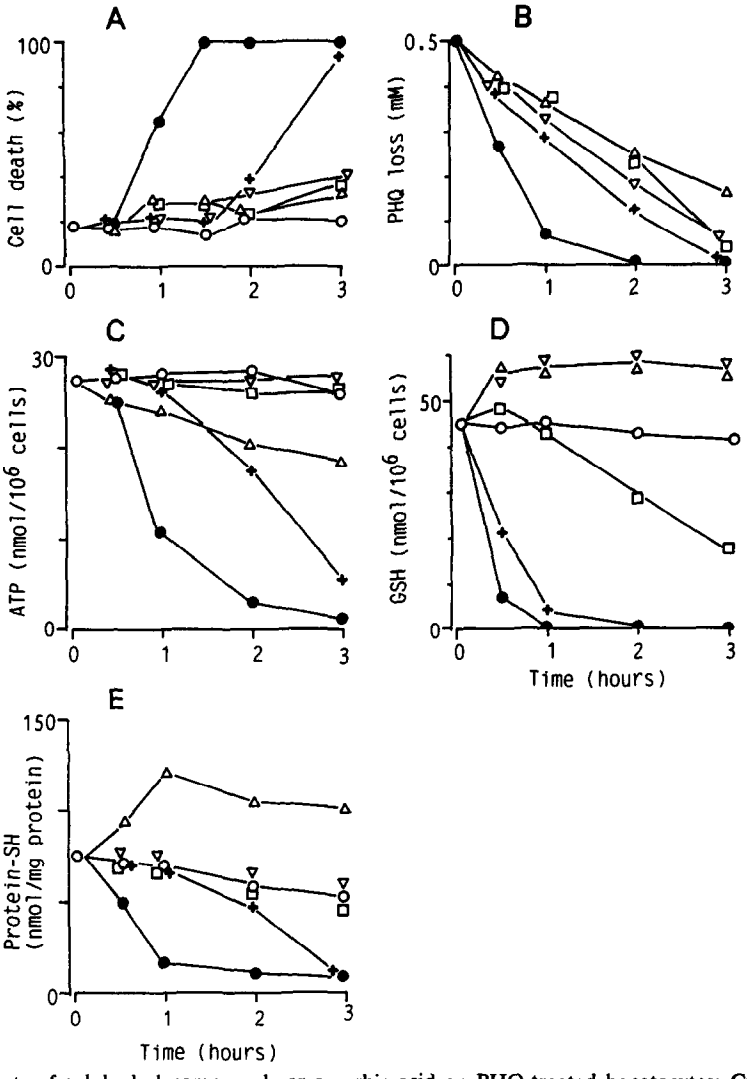

Fig. 5. Effects of sulphydryl compounds or ascorbic acid on PHQ-treated hepatocytes: Cytotoxicity (A), PHQ loss (B), and levels of ATP (C), GSH (D) and protein thiols (E). Hepatocytes (10^6 cells/mL) treated with 0.5 mM PHQ (●) were incubated in Krebs–Henseleit buffer supplemented with 5 mM of dithiothreitol (△), cysteine (□), *N*-acetyl-L-cysteine (▽) and ascorbic acid (+). (○) Untreated control hepatocytes. Results are expressed as the means from three experiments.

when the activity of microsomal monooxygenase is inhibited (Figs 1 and 2). Second, electrophilic intermediate(s) derived from OPP metabolism are involved in the cytotoxicity, since (1) OPP-induced cytotoxicity is enhanced by the depletion of GSH and (2) PHQ-induced cytotoxicity is prevented by sulphydryl compounds (Figs 4 and 5). On the basis of the present and previous studies [7, 11, 15], Fig. 6 shows a schematic outline of the relationship between OPP metabolites and cytotoxicity in isolated rat hepatocytes.

In previous experiments, we have demonstrated that addition of OPP (0.5–1.0 mM) to isolated rat hepatocytes causes dose-dependent cell death accompanied by the depletion of intracellular levels of ATP, GSH and protein thiols [15]. ATP loss induced by OPP is involved in serious impairment of mitochondrial functions related to oxidative phosphorylation and calcium flux [15]. In particular, a high dose (1.0 mM) of OPP elicits acute cell death (Fig. 1). Although the cells used previously differed from the hepatocyte system used here, OPP led to cytotoxicity of Chinese hamster ovary cells [25]. The 50% survival dose of OPP was approximately 0.6 mM in the absence of the S9 mixture [25]. These findings indicate that OPP exerts a direct toxic effect on cellular functions such as mitochondrial respiration.

OPP is oxidized by the microsomal cytochrome P450-dependent monooxygenase system to PHQ (Figs 2 and 3). PHQ is then converted by a non-enzymatic or enzymatic process to the corresponding PBQ via PHQ semiquinone [12, 14, 26]. Because the cytotoxicity induced by PHQ is indirectly correlated to PHQ loss (Fig. 5), PHQ itself may have some

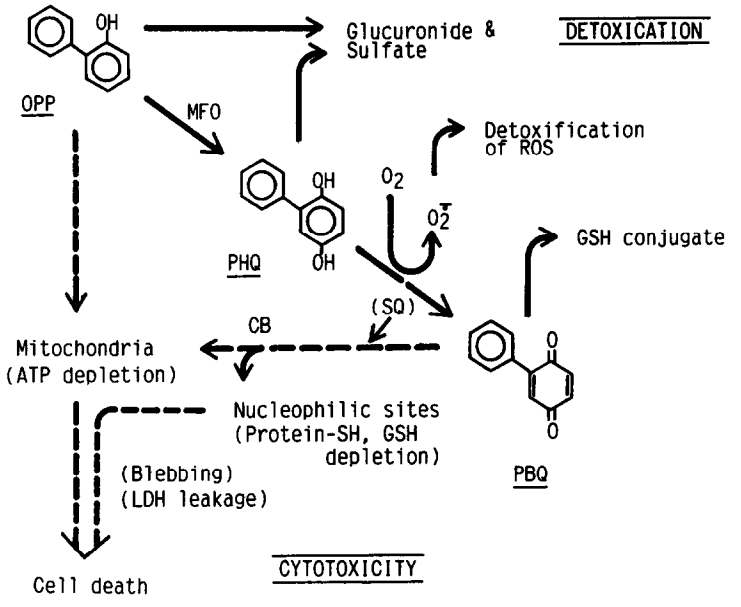

Fig. 6. Proposed mechanism of cytotoxicity induced by OPP and its metabolites in rat hepatocytes. Solid arrows indicate metabolic routes and dotted arrows indicate effects of OPP and its metabolites in hepatocytes. The abbreviations used are: MFO, mixed function oxidase; ROS, reactive oxygen species; SQ, semiquinone radical and CB, covalent binding.

toxic effects on hepatocytes. Actually, the inhibitory effects of PHQ on mitochondrial respiration were lower than those of either PBQ or OPP [15]. We have found that reactive intermediates derived from ^{14}C-labelled OPP irreversibly bind to proteins in the hepatic microsomal monooxygenase system [9]. It is well established that quinones and their semiquinones are highly reactive intermediates which react with nucleophilic groups such as cysteine, GSH, protein thiols and DNA [23, 24], and are potent inhibitors of a number of sulphydryl-dependent enzymes [27]. Although target sites of the electrophilic intermediates derived from OPP in hepatocytes are unclear, the protective agent, DTT can prevent completely the onset of PHQ-induced cell death (Fig. 5). The modification of protein thiols by arylation or oxidative stress is assumed to be a major factor in the tissue damage induced by some chemicals [28, 29]. Therefore, protein thiols may play a critical role in the onset of lethal alterations of cell function induced by OPP metabolism.

A single oral administration of OPP to rats has been shown to produce hepatic damage accompanied by depletion of tissue GSH [7]. This damage is potentiated by pretreatment with buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthesis [30]. In the present study, the depletion of cellular GSH with DEM potentiated the cytotoxicity induced by a low dose of OPP (Fig. 4). It is known that DEM enhances the microsomal aromatic hydroxylation [31] and the formation of PHQ derived from OPP is stimulated with 1.25 mM DEM (Fig. 4E). The prevention of PHQ-induced cytotoxicity by sulphydryl compounds suggests a correlation between toxicity and reactive intermediates present in the cells (Fig. 5). We find that PBQ reacts with GSH to produce a PHQ–GSH conjugate in phosphate buffer and that the conjugate is excreted into rat bile after oral administration of OPP [7, 9]. Indeed, the formation of the PHQ–GSH conjugate depends on both the cellular GSH concentrations and the amount of PHQ produced by the microsomal monooxygenase system (Figs 3 and 4). The availability of cellular GSH is, therefore, a critical factor that modulates the conjugation system for detoxification of electrophilic intermediates, such as PHQ semiquinone or PBQ.

Lipid peroxidation plays an important role in the onset of tissue damage induced by some chemicals [29]. It is known that the superoxide anion radical is produced by the oxidative pathway from PHQ to PBQ [10, 12]. Despite this, reactive oxygen species may not be involved directly in OPP-induced cytotoxicity since, first, OPP does not induce formation of malondialdehyde even if cellular GSH is consumed [15] and, second, there are antioxidant systems against the radicals in rat hepatocytes [32]. Recently, O'Brien [33] reported that most benzoquinones were cytotoxic as a result of alkylation whereas most naphthoquinones or phenanthraquinones mediated oxidative stress which could be cytotoxic [33]. Addition of ascorbic acid inhibits depletion of PHQ and delays the onset of cytotoxicity accompanied by loss of ATP and GSH (Fig. 5). Ascorbic acid can carry out a one-electron reduction of quinones and prevent the oxidation of hydroquinone [33]. From preliminary experiments, there is no evidence for conjugate reaction between PHQ and ascorbic acid. Therefore, ascorbic acid may merely reduce the oxidation of PHQ to PBQ.

In conclusion, this study demonstrates that OPP-induced cytotoxicity is enhanced by the inhibitor of microsomal cytochrome P450, SKF-525A, or by a depletion of GSH by DEM in hepatocytes. These results indicate that the acute cytotoxicity induced by a high dose of OPP (1.0 mM) is associated with the direct action of the parent compound and that the prolonged depletion of cellular GSH levels enhances the cytotoxicity induced by low concentrations of OPP metabolites. PHQ-induced cytotoxicity is prevented significantly by the addition of sulphydryl compounds or ascorbic acid.

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