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CYTOTOXICITY OF *ortho*-PHENYLPHENOL IN ISOLATED RAT HEPATOCYTES

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Abstract—The effects of *ortho*-phenylphenol (OPP) and its metabolites, phenyl-hydroquinol (PHQ) and phenyl-benzoquinone (PBQ), on isolated rat hepatocytes were investigated. Addition of OPP (0.5–1.0 mM) to cells caused a dose-dependent cell death accompanied by the depletion of intracellular levels of ATP, glutathione (GSH) and protein thiols. GSH loss correlated with the formation of oxidized GSH. In addition, PHQ and especially PBQ (both at 0.5 mM) resulted in acute cell death with rapid depletion of ATP, GSH and protein thiols, and further low doses of PBQ (10–50 μM) elicited serious impairment of mitochondrial functions related to oxidative phosphorylation and Ca fluxes in isolated liver mitochondria. These results indicate that mitochondria are a target for these compounds and that OPP is itself toxic to hepatocytes even when metabolism is inhibited. The loss of cellular GSH and protein thiols accompanied by the impairment of mitochondrial function may be the main mechanisms of cytotoxicity induced by OPP and its metabolites.

ortho-Phenylphenol (OPP§) and its sodium salt (SOPP) are broad spectrum antimicrobials with a variety of applications. They are used as fungicides and anti-bacterial agents in the post-harvest treatment of fruits. Consequently, OPP and SOPP have been extensively investigated 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, the chronic administration of large doses of OPP to rats caused renal damage [1] and bladder tumors [7]. Recently, we reported that a single large dose of OPP to rats produced acute hepatic and renal damage, exhibited as centrilobular or renal tubular necrosis, accompanied by depletion of tissue glutathione [8]. In addition, the damage was potentiated by pretreatment with buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase [8].

OPP and SOPP are oxidized by the microsomal monooxygenase system to PHQ (2,5-dihydroxybiphenyl) when conjugation processes of glucuronide and sulphate esters of the aromatic hydroxy group are saturated by higher concentrations of the compounds [9]. PHQ produced is converted to the corresponding PBQ via reactive semiquinone radicals. It is well established that quinone and semiquinone radicals are highly reactive intermediates which react with protein thiols and sulfhydryl compounds, such as cysteine and glutathione (GSH) and that they are potent inhibitors of a number of sulphydryl-dependent enzymes [10, 11]. The radioactive materials derived from ^{14}C-labelled OPP were irreversibly bound to macromolecules *in vivo* [12], and the radioactive OPP was bound covalently to microsomal protein by metabolism in a microsomal monooxygenase system [13]. In addition, PHQ was bound to calf thymus DNA in Tris–HCl buffer [14]. GSH conjugates existed in rat bile after oral administration of OPP *in vivo*, and were produced by OPP and GSH conjugates in the presence of microsomal monooxygenase system *in vitro* [13]. On the other hand, the toxicity of PBQ on rat liver or kidney was direct, and was greater than that of OPP or PHQ [8]. The hepatotoxicity induced by OPP may be related to the reactive intermediates and cellular glutathione levels and in this study, we report action of OPP and its metabolites on freshly isolated rat hepatocytes.

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.); glutathione reduced (GSH) and oxidized forms (GSSG), bovine serum albumin and collagenase (grade II) from the Sigma Chemical Co. (St Louis, MO, U.S.A.); 2,2-(1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bis(azo)dibenzenearsonic acid (Arsenazo III) and antimycin A from Boehringer (Mannheim, Germany). All other chemicals were of the highest grade of purity commerically available.

Isolation and incubation of hepatocytes. Male Fischer-344 rats (180–240 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of liver as described by Moldéus

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§ Abbreviations: OPP, *ortho*-phenylphenol; SOPP, sodium salt of OPP; PHQ, phenyl-hydroquinol; PBQ, phenyl-benzoquinone; GSH, reduced glutathione; GSSG, oxidized glutathione; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid); DMSO, dimethyl sulfoxide; MDA, malondialdehyde; RCI, respiratory control index.

et al. [15]. Cell viability was assayed by counting the percentages of the hepatocytes which excluded 0.16% Trypan blue and approximately > 90% of the fresh hepatocytes routinely excluded Trypan blue [15].

Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM Hepes. All incubations were performed in a rotating, round-bottomed flask at 37° under an atmosphere of 95% O_2 and 5% CO_2. Reactions were started by the addition of OPP or its metabolites dissolved in DMSO and aliquots of incubation mixture were taken at various times for the analyses of cell viability, cellular biochemical parameters and OPP and its metabolites.

Preparation of liver mitochondria. Liver mitochondria were isolated from male Fischer-344 rats (180–240 g) by differential centrifugation [16] in mediums containing (a) 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) and 1 mM EDTA for measurement of respiration rates or (b) 210 mM mannitol, 70 mM sucrose, 3 mM Hepes (pH 7.1) and 1 mM EDTA for measurement of Ca^{2+} release. EDTA was omitted in a final wash and resuspension. Protein concentration was determined by the Biurett method [17].

Measurement of respiration rates. The rate of oxygen consumption was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Model 53) at 25° in the presence (state 3) and absence (state 4) of 0.2 mM ADP [16]. Respiration buffer (3 mL) contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4) and 1 μM rotenone. The respiration substrate was 5 mM succinate and the amount of mitochondria was 1 mg protein/mL. The RCI was calculated as the ratio of state 3/state 4 respiration.

Measurement of mitochondrial Ca^{2+} *fluxes.* Ca^{2+} movements were measured by the absorbance change of Arsenazo III (49 μM), a metallochromic indicator for Ca^{2+}, in a Simadzu UV-3000 dual-wave length spectrophotometer using the wavelength pair 654–685 nm [18]. Mitochondria (1 mg protein/mL) were incubated at 25° in medium containing 210 mM mannitol, 70 mM sucrose, 3 mM Hepes (pH 7.4), 5 mM succinate and 3 μM rotenone. Mitochondria were loaded with calcium chloride (15 nmol/mg protein) for 1.5 min before the addition of OPP or its metabolites dissolved in DMSO. The final concentration of DMSO in all incubation mixtures was less than 0.5%. Antimycin A (5 μM) was added after OPP or its metabolites to ensure that these compounds did not interfere with the calcium measurement and to cause full release of calcium from mitochondria.

Biochemical assays. Adenine nucleotides in hepatocytes were measured by using HPLC following the procedure of Jones [19].

Cellular GSH and GSSG were determined by HPLC essentially as described by Reed *et al.* [20].

Reduced protein thiols were determined by Ellman's reagent (dithiobis dinitrobenzoic acid) as described previously [21].

MDA was measured as thiobarbituric acid-reactive products, as described previously [22]. The amount of reactive products formed was calculated by using an extinction coefficient of 156 $mM^{-1}\,cm^{-1}$ [23].

Blebbing of hepatocytes was assayed by light microscopy and expressed as the percentage of cells exhibiting multiple surface protrusions [24].

The activity of lactate dehydrogenase released from hepatocytes into the medium was determined by UV assay as described by Wroblewski and La Due [25].

RESULTS

The addition of OPP (0.5–1.0 mM) to isolated rat hepatocytes caused a concentration-dependent acute cell death (assessed by percentage Trypan blue uptake). This toxicity was accompanied by loss of cellular ATP, GSH and protein thiol levels (Figs 1 and 2). The appearance of surface blebs preceded the onset of cell death at 0.5–1.0 mM of OPP, and approximately 50% of viable cells treated with 0.5 mM OPP exhibited surface blebbing 3 hr later (Fig. 1B). Increase in lactate dehydrogenase leakage from cells correlated with loss of cell viability, but not with the formation of cell blebbing (Fig. 1A–C). The depletion of GSH was associated with the accumulation of GSSG (Fig. 2 A and B). In preliminary experiments, the increase in the GSSG level did not directly correlate with the activity of the GSH/GSSG redox cycling enzyme system, since the activities of both GSH peroxidase and GSH reductase were not inhibited by the concentration of OPP used (data are not shown). After a 1 hr incubation with 1.0 mM OPP, ATP and GSH were almost completely depleted in the hepatocytes (Figs 1 and 2). In these experiments, OPP is stable in Krebs–Henseleit buffer and the compound does not react with ATP or GSH in the absence of hepatocytes. As shown in Fig. 2D, cellular MDA accumulation was not affected by 0.5–1.0 mM of OPP.

PHQ is well known as a major metabolite of OPP; PHQ converts spontaneously to a reactive metabolite, PBQ [9, 13]. The amount of PHQ dissolved in Krebs–Henseleit buffer decreased with time and approximately 50% of PHQ was converted to PBQ by 30 min (data not shown). Studies were performed to evaluate the differences in cytotoxicity between OPP, PHQ and PBQ (all at 0.5 mM) (Fig. 3). The addition of PBQ to hepatocytes resulted in marked cytotoxicity and depletions of cellular ATP, protein thiols and GSH within 30 min. PHQ was much less hepatotoxic than PBQ, but still caused depletion of these parameters with time.

Table 1 shows the effects of OPP and its metabolites on the levels of adenine nucleotides in hepatocytes. The rapid disappearance of cellular ATP induced by 1.0 mM OPP or 0.5 mM PBQ was accompanied by a simultaneous increase in ADP and AMP levels 30 min later. Although 0.5 mM PBQ reduced the total adenine nucleotides pool to approximately 40% of controls, the pools in OPP- or PHQ-treated hepatocytes did not change markedly during the rapid loss of ATP. Since OPP or its metabolites do not affect the levels of ATP, ADP or AMP dissolved in Krebs–Henseleit buffer without hepatocytes for 30 min (data not shown), these results suggest that the depletion of ATP is due to

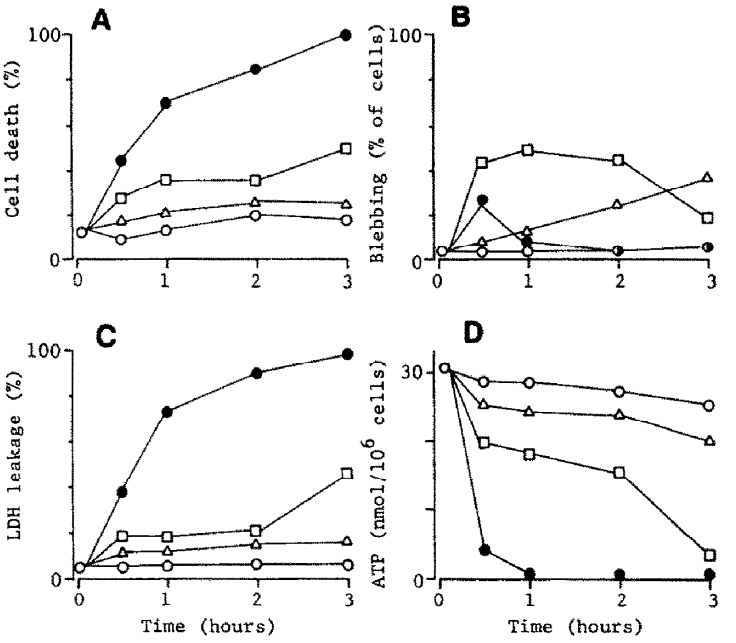

Fig. 1. Effects of OPP on cell viability (A), cell blebbing (B), lactate dehydrogenase leakage (C) and ATP levels (D) of isolated hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 0.5 mM (△), 0.75 mM (□) and 1.0 mM (●) of OPP, as described in Materials and Methods. Results are expressed as the means from three experiments.

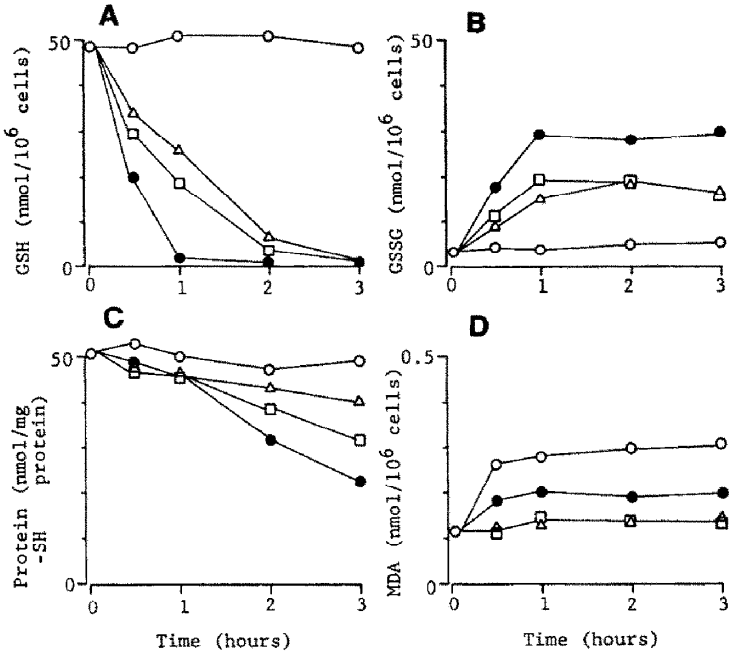

Fig. 2. Effects of OPP on levels of GSH (A), GSSG (B), protein thiols (C) and malondialdehyde (D) of isolated hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs–Henseleit buffer with no addition (○), 0.5 mM (△), 0.75 mM (□) and 1.0 mM (●) of OPP, as described in Materials and Methods. Results are expressed as the means from three experiments.

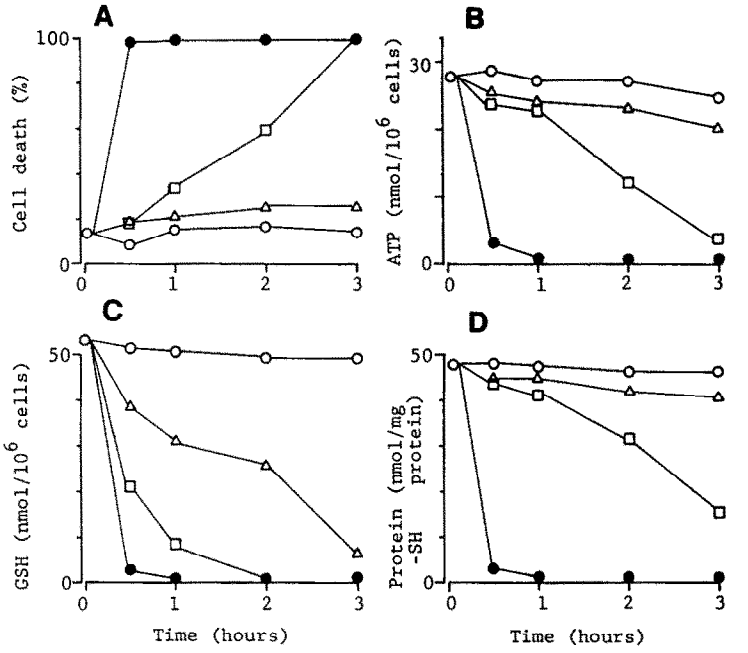

Fig. 3. Effects of OPP or its metabolites on cell viability (A) and levels of ATP (B), GSH (C) and protein thiols (D) of isolated hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs–Henseleit buffer with no addition (○), 0.5 mM OPP (△), 0.5 mM PHQ (□) and 0.5 mM PBQ (●) as described in Materials and Methods. Results are expressed as the means from three experiments.

Table 1. Effects of OPP or its metabolites on the level of adenine nucleotides in isolated hepatocytes

Incubation time (min)	Treatment (mM)		Adenine nucleotides (nmol/10^6 cells) ATP	ADP	AMP	Total
0	None		31.3	1.7	2.4	35.4
30	None		29.7	2.3	2.8	34.8
	OPP	0.50	25.4	6.2	5.3	36.9
		0.75	18.8	9.5	6.4	34.7
		1.00	3.6	12.9	15.4	31.9
	PHQ	0.50	26.8	2.9	3.4	33.1
	PBQ	0.50	2.4	3.3	14.9	20.6

Hepatocytes were incubated with OPP (0.5–1.0 mM), PHQ (0.5 mM) and PBQ (0.5 mM) for 30 min at 37° as described in Materials and Methods.

Values are the means from two experiments.

Table 2. Effects of OPP or its metabolites on mitochondrial respiration

Treatment (μM)		Mitochondrial respiration (ng atom O/mg protein/min) State 4	State 3	RCI
None		17.2 ± 1.4	73.2 ± 7.4	4.26
OPP	50	19.6 ± 0.5	51.9 ± 6.1	2.64
	100	22.7 ± 8.0	30.2 ± 7.0	1.38
	250	23.1 ± 0.9	20.8 ± 3.7	0.9
PHQ	500	16.9 ± 2.2	49.0 ± 2.9	2.90
	750	18.9 ± 3.0	33.1 ± 1.5	1.75
	1000	27.9 ± 3.7	28.9 ± 1.7	1.04
PBQ	10	24.8 ± 3.2	47.0 ± 4.6	1.90
	20	28.3 ± 3.7	29.3 ± 2.4	1.04
	50	3.6 ± 0.4	3.6 ± 0.3	1.00

Mitochondria (1 mg protein/mL) were preincubated in 3 mL of the respiration buffer, contained succinate (5 mM) and rotenone (1 μM), for 2 min at 25° (See Materials and Methods). For measurement of state 3 respiration, OPP or its metabolites was incubated with mitochondria for 2 min before the addition of ADP (200 μM). RCI was calculated as the ratio of state 3/state 4 respiration.

Values are the means ± SD from three experiments.

the inhibition of the adenine nucleotide synthesis system and/or the activation of hydrolysis of ATP by these compounds.

Inhibition of oxidative phosphorylation is one mechanism by which OPP or its metabolites can cause depletion of intracellular ATP levels. The effects of OPP or its metabolites on the oxygen consumption of isolated liver mitochondria is shown in Table 2, using concentrations of OPP, PHQ or PBQ that inhibited both state 3 respiration and stimulated state 4 respiration with 50 μM of PBQ. Therefore, inhibition of the RCI, a sensitive index of mitochondrial impairment, was due to both an inhibition of state 3 respiration and a stimulation of state 4 respiration. The effects of PBQ in particular on state 3 and 4 respirations were greater than those of either OPP or PHQ.

To evaluate further the effects of OPP or its

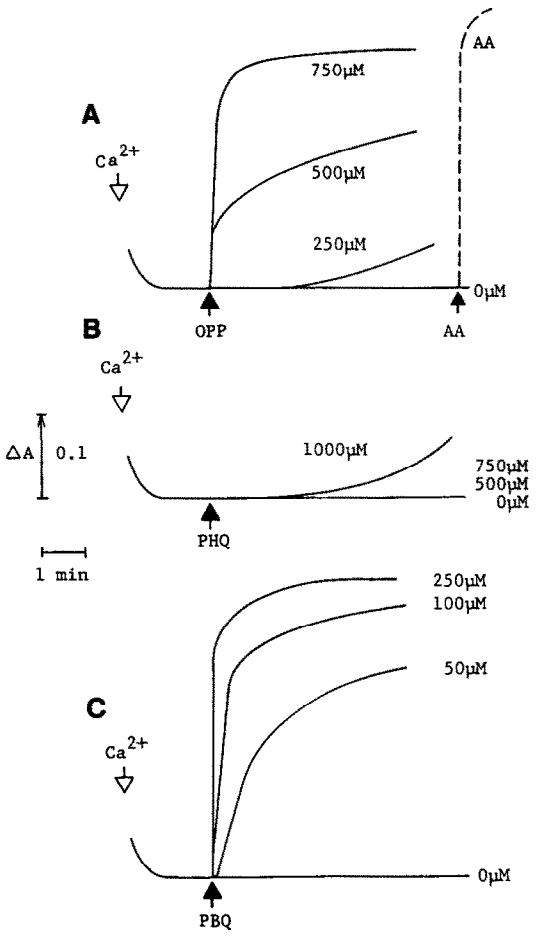

Fig. 4. OPP- or OPP metabolites-induced Ca^{2+} release from Ca^{2+}-loaded mitochondria. Mitochondria (1 mg protein/mL) were preincubated in a buffer medium, pH 7.4, supplemented with 5 mM succinate and 3 μM rotenone at 25° for 2 min. The mitochondria were then loaded with Ca^{2+} (15 nmol/mg protein) (open arrows) and after 2 min OPP (A), PHQ (B) and PBQ (C) were added at the indicated concentrations (closed arrows). Antimycin A (AA, 5 μM) was added as indicated.

metabolites on mitochondrial function, the flux of calcium in mitochondria was examined (Fig. 4). Calcium movements across the mitochondrial membrane were monitored by measuring calcium levels in the incubation medium using Arsenazo III. Addition of OPP (250–750 μM), PHQ (500–1000 μM) or PBQ (50–250 μM) to Ca^{2+}-loaded isolated mitochondria resulted in a concentration-dependent Ca^{2+} release (Fig. 4). OPP (at concentrations higher than 500 μM) and PBQ (at concentrations higher than 50 μM) caused a rapid Ca^{2+} release from mitochondria immediately after addition to the incubation mixtures, 1.0 mM PHQ produced only a slow Ca^{2+} release from mitochondria. Again, PBQ was the most potent followed, in decreasing order of potency, by OPP and PHQ.

DISCUSSION

The results obtained in the present study indicate that OPP and/or its metabolites, PHQ and PBQ, are cytotoxic for isolated rat hepatocytes. These compounds cause dose-dependent depletion of intracellular levels of ATP and GSH, which consistently precede cell death. In addition, OPP and its metabolites, especially PBQ, result in the serious impairment of mitochondrial functions related to oxidative phosphorylation and calcium flux. PBQ was most toxic, followed by PHQ and OPP. Further, the toxic potency of these compounds found in hepatocytes is similar to the *in vivo* findings of hepatic or renal damage in rats [8].

In previous experiments, oral administration of OPP to rats resulted in rapid loss of hepatic GSH level preceded by centrilobular necrosis; PHQ–GSH conjugates derived from activated metabolites of OPP were found in bile [8, 13]. GSH is well known to play a crucial protective role against cellular injury caused by a number of toxic compounds [26]. Addition of exogenous GSH or other thiol compounds prevented OPP- or OPP metabolites-induced cyto- and geno-toxicity in CHO K1-cells with the S9 mixture system [3]. Despite this, pretreatment with L-buthionine-*S*,*R*-sulfoximine, an inhibitor of GSH synthesis, enhanced OPP-induced hepatic damage in rats [8]. For the protection of hepatocytes from the toxic effects of reactive metabolites, the conjugation with GSH has a more direct implication since the cytotoxicity induced by OPP was accompanied by acute depletion of GSH (Fig. 2) and the formation of PHQ–GSH conjugates (data not shown). Although it is not clear what mechanism ultimately leads to rapid formation of GSSG by high concentrations (1.0 mM) of OPP, there is the possibility that OPP-induced toxicity is related to PHQ and/or PBQ and that following depletion of GSH, some of these intermediates interact with GSH and protein thiols.

It is well known that a loss of protein thiols following GSH loss is cytotoxic for isolated hepatocytes [21, 22, 24, 27]. The levels of cellular protein thiols are another toxic parameter related to GSH and GSSG status [28]. Several mechanisms have been considered to account for depletion of protein thiols: first, the toxic compound or its reactive metabolite(s) may react with protein thiols. Quinones such as PBQ are electrophilic and react directly with thiols resulting in a loss of protein thiols of GSH by arylation. Second, an increase in the content of GSSG may stimulate formation of mixed disulfides. In preliminary experiments, we found that the rapid increase in GSSG levels induced by OPP was not related directly to the impairment of the enzyme systems of the GSH/GSSG redox cycling, since both activities of glutathione peroxidase and glutathione reductase were unaffected by treatment with 0.5–1.0 mM OPP (data not shown).

Lipid peroxidation and subsequent cellular damage are regarded as an important mechanism underlying the toxicity of several xenobiotics [29, 30]. However, this does not play a role in OPP-induced cytotoxicity since no lipid peroxidation was observed in hepatocytes treated with 1.0 mM OPP even though most of the GSH and some of the protein thiols were depleted. For these reasons, it may be associated with a multicomponent antioxidant

system, which includes various enzymes, ascorbate, α-tocopherol and other cellular reductants, even when GSH is depleted completely [31], as well as phenolic compounds with antioxidant properties [32].

Because cytotoxicity involves mitochondrial impairment by the respiratory processes and subsequent depletion of cellular energy supplies in the form of ATP [16, 33, 34], we monitored cellular ATP levels and some mitochondrial physiological functions (Figs 1, 3 and 4; Tables 1 and 2) and found mitochondria to be a primary target when isolated hepatocytes are exposed to OPP or its metabolites. In isolated mitochondria, the order of potency for respiration or Ca^{2+} release was PBQ > OPP > PHQ. Thus, the parent compound itself has an inhibitory action on mitochondrial function. The depletion of cellular ATP levels affects many cellular activities, including the ability of cells to maintain ionic gradients of calcium through the function of ATP-dependent translocases. To account for chemically induced Ca^{2+} release from mitochondria, several mechanisms have been proposed, including non-specific membrane damage [35], oxidation of protein thiols [36] and oxidation of pyridine nucleotides [37, 38]. Indeed, some quinones caused mitochondrial Ca^{2+} release accompanied by changes in the NAD(P)H/NAD(P) redox status [18, 36]. The increase in blebbing cells induced by OPP (Fig. 1B) may be related to a disturbance of intracellular Ca^{2+} homeostasis, since it has been proposed that drug-induced hepatotoxicity is associated by an increased cytosolic Ca^{2+} concentration accompanied by the formation of blebbing of cell surface [39, 40]. Further investigations are necessary to determine the mechanisms of mitochondrial impairment induced by OPP or its metabolites.

In conclusion, this study demonstrates that OPP and its metabolites cause acute cytotoxicity in isolated rat hepatocytes. A combination of depletion of cellular GSH and protein thiols, and of induction of GSSG and impairment of mitochondrial oxidative phosphorylation appear to be the main mechanisms of the cytotoxicity.

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