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CYTOTOXIC EFFECTS OF PHENYL-HYDROQUINONE AND SOME HYDROQUINONES ON ISOLATED RAT HEPATOCYTES

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Abstract—The cytotoxic effects of phenyl-hydroquinone (PHQ) and some other hydroquinones on freshly isolated rat hepatocytes were investigated. Addition of PHQ (0.5 or 0.75 mM) to the hepatocytes elicited dose-dependent cell death accompanied by losses of intracellular glutathione (GSH), protein thiols and ATP. These effects were related to both PHQ loss and phenyl-benzoquinone (PBQ) formation in the cell suspension. The cytotoxicity of PHQ was prevented by sulphydryl compounds such as cysteine and GSH. In Krebs–Henseleit buffer without cells, loss of PHQ (0.5 mM; initial concentration) and formation of PBQ, monitored by spectral measurements, were inhibited by addition of 50 μM GSH. Further, the oxygen consumption owing to autoxidation of PHQ (0.5 mM) in Krebs–Henseleit buffer without cells was depressed by addition of 50 μM GSH. Among all the hydroquinones tested (at 0.5 mM), *tert*-butyl-hydroquinone and PHQ were most toxic, followed by hydroquinone and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone. However, accumulation of cellular malondialdehyde was not affected by these hydroquinones. The toxicity was related to the rate of oxygen consumption by each hydroquinone in the buffer. These results suggest that hydroquinone-induced cytotoxicity is dependent on the rate of oxidation of these compounds as well as the loss of protein thiols.

Phenyl-hydroquinone (PHQ‡) is a major metabolite formed from ortho-phenylphenol (OPP) and its sodium salt by the action of the microsomal monooxygenase system. The parent compounds are used as fungicides and anti-bacterial agents in the post-harvest treatment of fruits and vegetables. It is well known that hydroquinones are converted to semiquinones and benzoquinones by autoxidation with formation of a superoxide anion radical, which causes oxidative stress to cells. Both quinones are highly reactive intermediates which react with nucleophilic groups in various cellular components [1, 2]. In previous experiments, some intermediates derived from OPP were shown to be irreversibly bound to cellular macromolecules and nucleophilic groups *in vivo* and *in vitro* [3]. Although the exact mechanisms of the various OPP-induced toxicities in rat liver and bladder [4, 5], isolated rat hepatocytes [6, 7] and Chinese hamster ovary (CHO-K1) cells [8, 9] remain unclear, metabolic activation of OPP has been postulated to be associated with these toxicities. As regards toxicity to rat liver and kidney and isolated hepatocytes, PBQ was most toxic followed by PHQ and OPP [4, 6]. However, autoxidation of PHQ leads to PBQ and the superoxide anion radical via reactive semiquinone radicals [10]. PBQ reacts rapidly with GSH in alcohol-phosphate buffer and produces PHQ–GSH conjugates [3]. These conjugates were found in bile after oral administration of OPP in rats [3]. In addition, PHQ binds to calf thymus DNA in Tris–HCl buffer [11]. Therefore, free unconjugated PHQ produced by the microsomal monooxygenase system may play an important role in the initiation of OPP-induced toxicity. In the present study, we investigated the cytotoxic actions of PHQ and some other hydroquinones on freshly isolated rat hepatocytes.

MATERIALS AND METHODS

Materials. The chemical compounds utilized were purchased from the following companies: PHQ, PBQ and 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (ditBHQ) (purities >97%) from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); hydroquinone (HQ) and *tert*-butyl-hydroquinone (tBHQ) (purities >99%) from the Tokyo Kasei Co. (Tokyo, Japan); reduced glutathione (GSH) and its oxidized form (GSSG), and bovine serum albumin from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and collagenase from Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals were of the highest purity commercially available. Chemical structures of the hydroquinones used are shown in Fig. 1.

Isolation and incubation of hepatocytes. Male Fischer-344 rats (220–280 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion on liver as described by Moldéus *et al.* [12]. Viability of hepatocytes was assessed by using trypan blue. Approximately 90% of freshly isolated hepatocytes routinely excluded trypan blue.

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‡ Abbreviations: OPP, *ortho*-phenylphenol; PHQ, phenyl-hydroquinone; PBQ, phenyl-benzoquinone; HQ, hydroquinone; tBHQ, *tert*-butyl-hydroquinone; ditBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; GSH, reduced glutathione; GSSG, oxidized glutathione; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid); MDA, malondialdehyde.

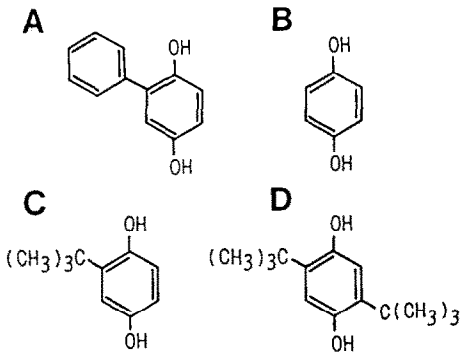

Fig. 1. Chemical structures of hydroquinones utilized in this study. (A) PHQ, (B) HQ, (C) tBHQ, (D) ditBHQ.

Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM Hepes and 0.1% albumin. All incubations were performed in rotating, round-bottomed flasks at 37° under a constant flow of humidified 95% O_2 and 5% CO_2. In the experiments with added sulphydryl compounds, 5 mM GSH or cysteine was dissolved in Krebs–Henseleit buffer and added to the cell suspension 5 min prior to PHQ treatment. Reactions were initiated by the addition of PHQ or other hydroquinones dissolved in dimethyl sulfoxide (final concentration less than 1%). Aliquots of the cell suspension were taken at definite intervals for the determination of cell death, as well as for quantification of the concentrations of GSH, GSSG, ATP, protein thiols, protein, malondialdehyde, PHQ and PBQ.

Biochemical assays. ATP concentration in hepatocytes was measured by using HPLC according to the procedure of Jones [13].

Cellular GSH and GSSG levels were determined by HPLC essentially as described by Reed *et al.* [14].

Reduced protein thiol concentrations were determined by using Ellman's reagent (dithiobis-(dinitrobenzoic acid)) as described previously [15].

Protein was measured by the method of Lowry *et al.* [16] using serum albumin as a standard.

Malondialdehyde (MDA) was measured as thiobarbituric acid-reactive products as described previously [17].

Change in UV-visible spectra or the rate of oxygen consumption by PHQ in Krebs–Henseleit buffer was measured with a spectrophotometer (Shimadzu Co., Kyoto, Japan, Model 250) or polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Model 5300) at 30°.

Determination of PHQ and PBQ by HPLC. Aliquots of hepatocyte cell suspensions were treated with a cell disrupter (Sonifier, Branson Sonic Power Co., Danbury, CT, U.S.A.) in ice water for 20 sec and then filtered through a membrane cartridge (pore size 0.45 μm). The eluate was injected onto an analytical TSKgel ODS-120T column (4.6 mm i.d. × 250 mm, Toyo Soda Co., Tokyo, Japan) 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.

RESULTS

Addition of PHQ (0.50 or 0.75 mM) to freshly isolated hepatocytes caused concentration-dependent cell death accompanied by the depletion of intracellular levels of GSH, protein thiols and ATP (Fig. 2 A–D). The cellular level of GSH was depleted rapidly by PHQ, prior to the decrement of protein thiols and ATP. In addition, cell death was associated with the loss of PHQ and the formation of PBQ (Fig. 2 E, F). Accumulation of cellular MDA was not seen upon addition of PHQ (0.5–0.75 mM) during the incubation period (data not shown). These results demonstrate a correlation between the onset of cytotoxicity and the formation of PBQ.

As PHQ, a major metabolite of OPP, is spontaneously converted to PBQ via the semiquinone radicals and PBQ reacts with GSH to form the corresponding conjugates [3, 10], the effects of sulphydryl compounds on PHQ-induced cytotoxicity were investigated (Fig. 3). The cell death induced by PHQ (0.50 mM) was significantly reduced by addition of 5 mM GSH or cysteine during the experimental period. When PHQ was added to a cell suspension pretreated with GSH, the amount of the PHQ–GSH conjugate increased with time (data not shown). In addition, the rates of loss of PHQ, protein thiols and ATP were decreased significantly by addition of either of these sulphydryl compounds.

The rates of PHQ loss and/or PBQ formation were measured following the addition of a cellular level (50 μM; average level based on 50 nmol/10^6 cells/mL buffer) of GSH to a solution of 0.5 mM PHQ dissolved in Krebs–Henseleit buffer without hepatocytes (Fig. 4). The changes in the concentration of PHQ and PBQ were monitored by measuring optical density; the absorption maxima in the buffer were at 300.1 nm and 373.7 nm, respectively. The loss of PHQ and formation of PBQ were inhibited simultaneously by the addition of GSH. In contrast, addition of 20 μM ascorbic acid (average level in hepatocytes [18]) to the buffer containing PHQ did not affect the loss of PHQ. The oxygen consumption induced by autoxidation of PHQ in Krebs–Henseleit buffer was inhibited significantly by the addition of GSH. Oxygen consumption with 0.5 mM PHQ alone and with 0.5 mM PHQ and 50 μM GSH in the buffer were 320.1 ± 13.8 and 89.1 ± 5.5 ng atom oxygen/15 min (means ± SD from three measurements), respectively. At the end of the reaction, addition of catalase (10 U/mL) and superoxide dismutase (10 U/mL) to the reaction mixture resulted in the regeneration of oxygen. The results suggest that the oxygen consumption in the buffer is due to autoxidation of PHQ coupled to formation of superoxide anion radicals.

The cytotoxic effects of PHQ, HQ, tBHQ and ditBHQ were studied on isolated hepatocytes (Fig. 5) PHQ and tBHQ were more toxic than HQ or ditBHQ. Although cell death from PHQ, tBHQ and HQ was associated with depletion of ATP, GSH and protein thiols, rapid loss of ATP caused by

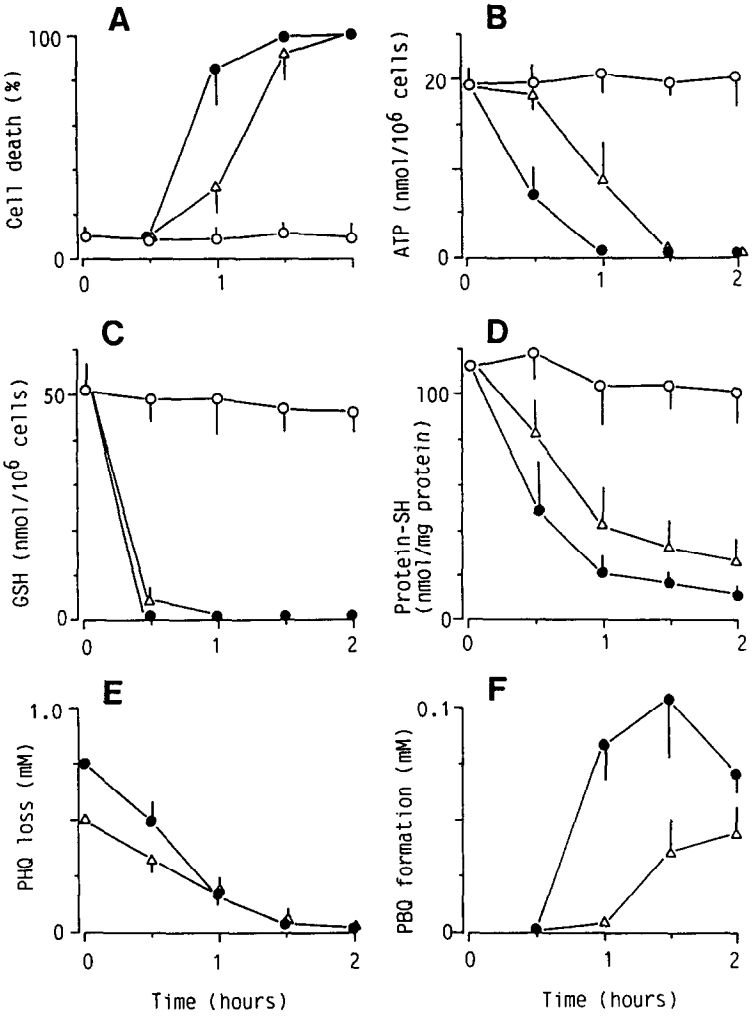

Fig. 2. Effects of PHQ on cell viability (A) and levels of ATP (B), GSH (C), protein thiols (D), PHQ (E), and PBQ (F) of isolated hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 0.5 mM (△) and 0.75 mM (●) of PHQ, as described in Materials and Methods. Results are expressed as the means ± SE from three experiments.

ditBHQ was not directly related to the onset of cell death. The toxicity was correlated with the rate of oxygen consumption by these compounds in Krebs–Henseleit buffer without hepatocytes (Fig. 6). In the case of treatment with tBHQ or HQ, however, there was not a good correlation between cytotoxicity and oxygen consumption, since oxygen consumption with both hydroquinones was similar (Fig. 6), but tBHQ was more toxic than HQ (Fig. 5). At the end of the reaction (indicated by closed arrows), oxygen was regenerated by the addition of catalase (10 U/mL) and superoxide dismutase (10 U/mL) to the reaction mixture (Fig. 6). Although superoxide anion radicals were generated by autoxidation and these hydroquinones except ditBHQ, these compounds did not induce the accumulation of cellular MDA during the incubation period (data not shown). The onset of cytotoxicity induced by these hydroquinones may be related to the formation of quinones rather than to lipid peroxidation.

DISCUSSION

In previous studies [6, 7], we demonstrated that at least two mechanisms are involved in OPP-induced cytotoxicity. The first mechanism is the disturbance of mitochondrial respiration by the direct action of the parent compound, OPP. The second mechanism is through interactions between some intermediates derived from OPP and mitochondrial and other cellular functions. The results of this study indicate that PHQ, a major metabolite of OPP produced by the microsomal monooxygenase system, elicits cell death via the formation of electrophilic intermediates, since the cytotoxicity is closely related to the accumulation of PBQ in the cell suspension (Fig. 2A, F). PBQ at concentrations higher than 50 μM causes serious impairment of oxidative phosphorylation, as well as calcium flux, in isolated mitochondria [6]. Indeed, the inhibitory effects of PHQ on mitochondrial respiration and

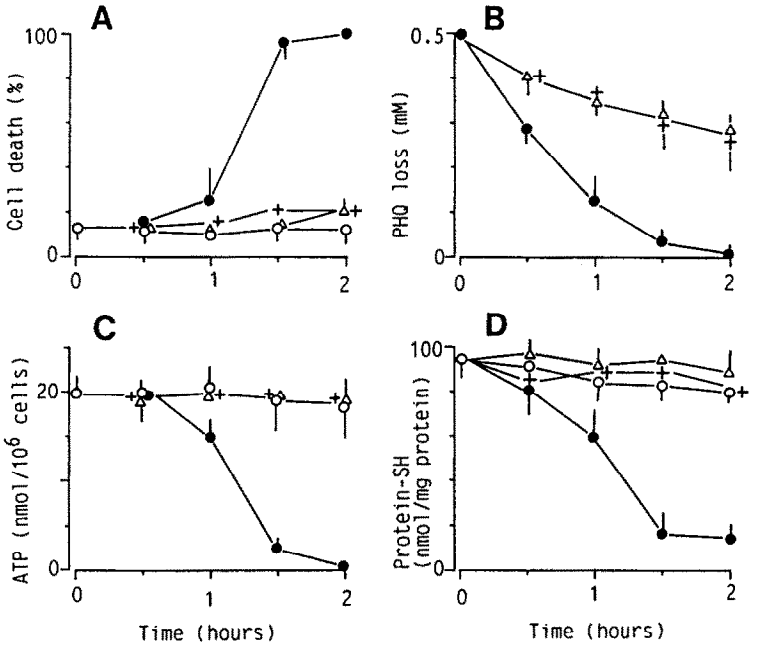

Fig. 3. Effects of GSH or cysteine on PHQ-treated hepatocytes: cell viability (A), PHQ loss (B), and levels of ATP (C) and protein thiols (D). Hepatocytes (10^6 cells/mL) treated with 0.5 mM PHQ (●) were incubated in Krebs–Henseleit buffer supplemented with 5 mM of GSH (△) and cysteine (+). (○) Untreated control hepatocytes. Results are expressed as the means ± SE from three experiments.

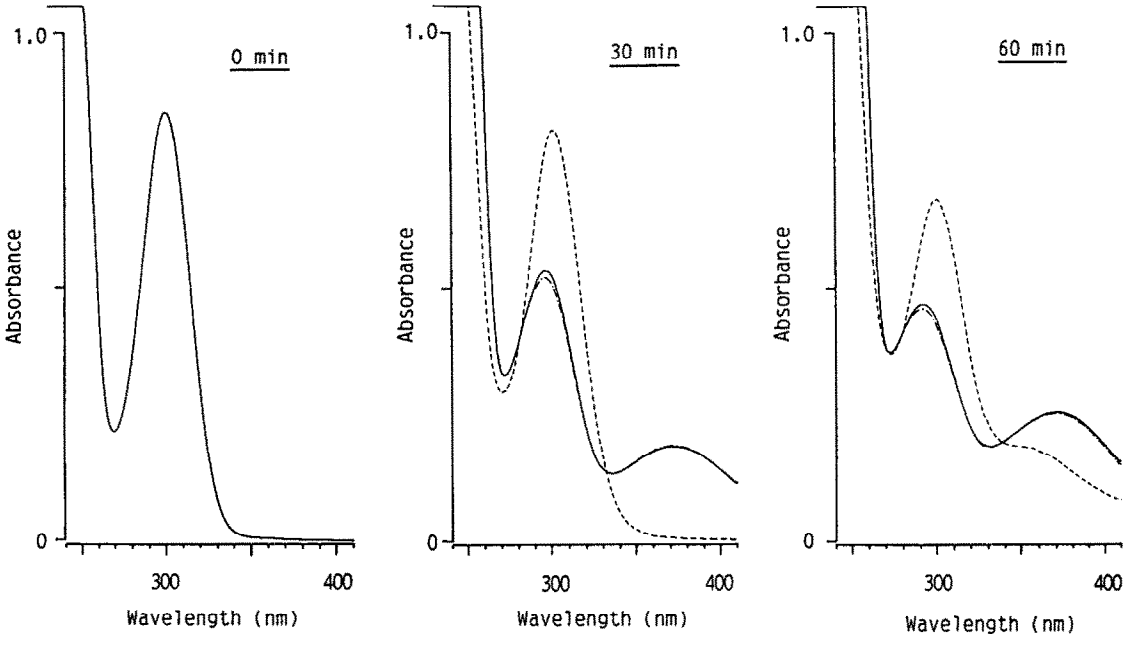

Fig. 4. Time-course of changes in absorption spectra of PHQ and PHQ plus GSH or ascorbic acid. The spectrum of 0.5 mM PHQ (—) dissolved in Krebs–Henseleit buffer (pH 7.4) at 37° was monitored with time after addition of 50 μM GSH (----) or 20 μM ascorbic acid (—·—). At definite time, each mixture was diluted three times with the buffer and then was scanned from UV to visible region. Absorption maxima of PHQ and PBQ were at 300.1 and 373.7 nm, respectively.

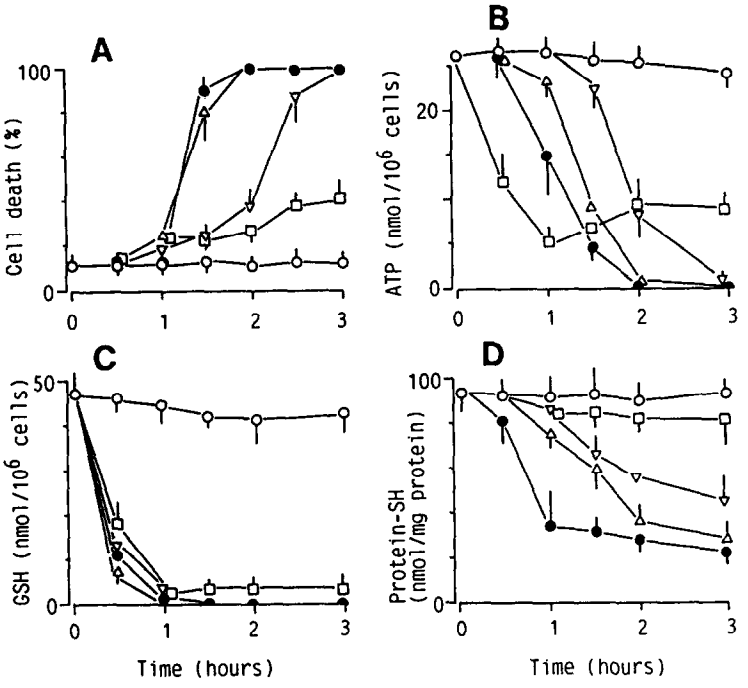

Fig. 5. Effects of PHQ and other hydroquinones on cell viability (A), and levels of ATP (B), GSH (C) and protein thiols (D) of isolated hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with 0.5 mM of PHQ (●), tBHQ (△), HQ (▽) and ditBHQ (□) in Krebs–Henseleit buffer, pH 7.4, as described in Materials and Methods. (○) Untreated control hepatocytes. Results are expressed as the means ± SE from three experiments.

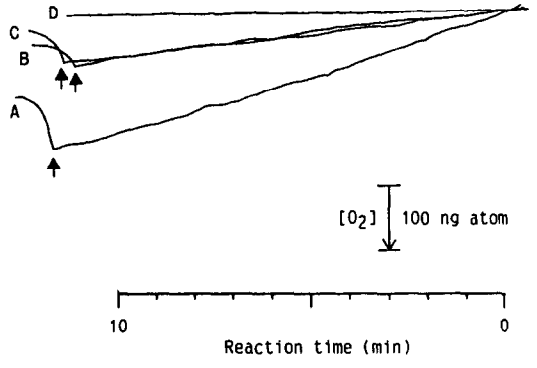

Fig. 6. Oxygen consumption with PHQ (A), tBHQ (B), HQ (C) and ditBHQ (D) in Krebs–Henseleit buffer, pH 7.4, at 30°. Each hydroquinone (final 0.5 mM) dissolved in dimethyl sulphoxide was added to the buffer (total 3 mL) and, after 12 min (at closed arrows), superoxide dismutase (30 U) and catalase (30 U) were added to the reaction mixture.

calcium flux were much less than those of PBQ or the parent compound [6]. Thus, the onset of PHQ toxicity depends on the rate of PBQ formation. When cellular GSH and the conjugation systems for glucuronides and sulphates are maintained at normal levels, PHQ itself may be conjugated and excreted in a process of detoxication.

PHQ-induced cytotoxicity is prevented by the addition of sulphydryl compounds such as GSH and cystein (Fig. 3). In our previous studies, PBQ or PHQ semiquinone reacted with GSH to produce GSH conjugates in alcohol-phosphate buffer [3]. These conjugates were found in rat bile after oral administration of OPP [3, 4]. It is well established that the availability of cellular GSH is critical for electrophilic intermediates produced by various chemicals [19–22]. In cell-free Krebs–Henseleit buffer, 50 μM GSH (average level of hepatocytes) prevents significant oxidation of 0.5 mM PHQ to the corresponding quinone PBQ for 30 min, without the formation of GSH conjugates (Fig. 4). Although it is known that ascorbic acid can cause one-electron reduction of quinones and prevent the oxidation of hydroquinone [23], intracellular concentrations (approximately 20 μM [18]) of ascorbic acid did not inhibit the oxidation of PHQ (0.5 mM). This result indicates that GSH rather than ascorbic acid acts as an important reductant to prevent PHQ autoxidation.

Although hydroquinones such as HQ, tBHQ and PHQ react with GSH to form the corresponding GSH conjugates [3, 24, 25], it has not been observed that ditBHQ forms GSH conjugates. To explain why ditBHQ does not react with GSH, Moore *et al.* [26] suggested that the presence of two *tert*-butyl groups on the phenol ring sterically hinders addition

reactions with GSH. The cytotoxicity induced by ditBHQ may be the result of a perturbation of intracellular Ca^{2+} homeostasis, since this compound is a potent and selective inhibitor of liver microsomal Ca^{2+} sequestration [26], and increased cytosolic Ca^{2+} concentrations precede the onset of cell death induced by some chemicals and by oxidative stress [27]. It has also been proposed that the elevation of cytosolic free Ca^{2+} by some chemicals causes cytotoxicity with cell blebbing, which precedes cell death [28]. In fact, numerous blebs on the plasma membrane were found in approximately 40% of hepatocytes 60 min after the addition of ditBHQ (data not shown), but Ca^{2+} sequestration and F_1F_0-ATPase of mitochondria are not affected [26]. Intracellular ATP levels during the incubation period are held at approximately one-third of the control level after an initial decrease resulting from the addition of ditBHQ (Fig. 5B). In contrast, PHQ, tBHQ and HQ are more toxic to hepatocytes when compared with ditBHQ at the concentration of 0.5 mM. The relative cytotoxicity is dependent on the rate of autoxidation of each compound and the resultant cellular GSH depletion by oxidative stress via superoxide anion radical formation (Figs 5 and 6). These results suggest that ditBHQ has a mechanism of cytotoxicity different from that of the other hydroquinones.

It is well known that lipid peroxidation and subsequent cellular damage are important mechanisms of action for several chemicals [29]. However, superoxide anion radicals produced by autoxidation of these hydroquinones are not directly responsible for the onset of cytotoxicity, since lipid peroxidation as assessed by MDA accumulation was not observed in cell suspensions treated with these hydroquinones during the incubation period. The absence of MDA production can be accounted for by the fact that there are effective multi-antioxidant systems that prevent oxidative stress in normal hepatocytes [18]. Despite this, both the alkylation of critical protein thiols by compounds or metabolites and the formation of mixed disulphides induced by accumulation of GSSG within the cell are assumed to be major factors in tissue damage [30]. Indeed, GSH and cysteine can protect cells from both cytotoxicity and depletion of protein thiols induced by PHQ (Fig. 3). The depletion of protein thiols in hepatocytes correlates with the onset of cytotoxicity induced by tBHQ or HQ (Fig. 5). Therefore, the level of cellular protein thiols is presumed to be an important parameter in hydroquinone-induced cytotoxicity.

In conclusion, this study using freshly isolated hepatocytes demonstrates that cell death induced by PHQ correlates with PBQ formation, which is accompanied by rapid depletion of intracellular GSH, protein thiols and ATP. The PHQ-induced cytotoxicity is prevented by the addition of sulphydryl compounds. PHQ, tBHQ and HQ are more toxic than ditBHQ, and the degree of their toxicity may be related to the rate of oxidation of these compounds, as well as the depletion of protein thiols.

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