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SHORT COMMUNICATION

Role of Mitochondrial Membrane Permeability Transition in *p*-Hydroxybenzoate Ester-Induced Cytotoxicity in Rat Hepatocytes

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ABSTRACT. The relationship between mitochondrial membrane permeability transition (MPT) and the toxic effects of the alkyl esters of *p*-hydroxybenzoic acid (parabens) has been studied in mitochondria and hepatocytes isolated from rat liver. MPT has been proposed as a common final pathway in acute cell death through mitochondrial dysfunction. In isolated mitochondria, propyl-paraben (0.1 to 0.5 mM) in the presence of Ca^{2+} (50 μM) elicited a concentration-dependent induction of mitochondrial swelling dependent on MPT. This was prevented by pretreatment with a specific inhibitor of MPT, cyclosporin A (0.2 μM). For the other parabens tested, the induction of MPT depended on the relative elongation of alkyl side-chains in their molecular structure and was associated with the partition coefficients. In contrast, the induction caused by *p*-hydroxybenzoic acid was more potent than that of methyl- or ethyl-paraben. The pretreatment of freshly isolated hepatocytes with cyclosporin A (5 μM) and trifluoperazine (10 μM), which inhibit MPT in a synergistic manner, partially but not completely prevented propyl-paraben (1 mM; plus diazinon, 100 μM)-induced cell death, ATP loss, and decreased mitochondrial membrane potential. These results suggest that the onset of paraben-induced cytotoxicity is linked to mitochondrial failure dependent upon induction of MPT accompanied by the mitochondrial depolarization and depletion of cellular ATP through uncoupling of oxidative phosphorylation. BIOCHEM PHARMACOL **58**;5:811–816, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *p*-hydroxybenzoate esters; parabens; mitochondrial permeability transition; mitochondrial dysfunction; cytotoxicity; antimicrobial preservative

Alkyl esters of *p*-hydroxybenzoic acid, which are commonly called parabens§, are used widely as antimicrobial preservatives in pharmaceuticals, cosmetic products, processed foods, and beverages. Because of their widespread use, the potential toxicity and metabolic disposition of parabens have been investigated *in vivo* and *in vitro* to assess various toxicological and pharmacological properties [1–5]. Despite their presumed low toxicity and the known information about their disposition, studies have not investigated the relationship between metabolism and the cytotoxic effects of parabens. A previous study [6] has shown that hydrophobic parabens with elongation of the alkyl side-chain are cytotoxic. Experiments with isolated hepatocytes and liver mitochondria indicate that this is most likely mediated through impairment of mitochondrial function accompanied by failure of oxidative phosphorylation and mitochondrial membrane depolarization, which are consequent on decreased cellular levels of adenine nucleotides. A number of observations suggest that mitochondria are a primary target of chemical-induced injury and that their dysfunction ultimately leads to cell death [7]. For instance, an insufficient supply of ATP limits activities of all energy-requiring reactions and eventually leads to cell death. Induction of MPT, an abrupt increase in the permeability of the inner mitochondrial membrane to small molecular weight solutes, has been proposed to be an important common pathway causing cellular injury. A direct consequence of MPT is that mitochondria are no longer able to maintain energy generation [8–10]. It has been reported that the induction of MPT through opening of high-conductance pores, which are specifically blocked by cyclosporin A, induces mitochondrial depolarization, uncoupling of oxidative phosphorylation with ATP depletion, and ultimately cell death [11, 12]. Because it is possible that parabens, like some other chemicals, induce a calcium-dependent increase in permeability of the mitochondrial inner membrane, in the present study we investigated (i) the effect of parabens on MPT in isolated liver mitochondria, and (ii) the possible role of the MPT in paraben-

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§ *Abbreviations:* parabens, alkyl esters of *p*-hydroxybenzoic acid; diazinon, phosphorothioic acid O,O-diethyl-O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl]ester; MPT, mitochondrial membrane permeability transition; and MOPS, 3-[*N*-morpholino]propanesulfonic acid.

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induced cell damage in isolated hepatocytes. The mechanism of the toxic effects of parabens is discussed.

MATERIALS AND METHODS

Materials

The chemical compounds used were obtained from the following companies: *p*-hydroxybenzoic acid and its esters (parabens): methyl-, ethyl-, propyl-, and butyl-*p*-hydroxybenzoates (purities of > 99%) from the Tokyo Kasei Co.; adenine nucleotide, bovine serum albumin, ruthenium red, rhodamine 123, MOPS, and trifluoperazine from the Sigma Chemical Co.; and collagenase and cyclosporin A from Wako Chemicals. All other chemicals were of the highest purity commercially available.

Isolation and Incubation of Hepatocytes

Male F344/DuCrj (240–270 g) rats were obtained from Charles River Japan Inc. and were allowed food (CE-2, Clea Japan Inc.) and water *ad lib*. before hepatocytes were prepared. The hepatocytes were isolated by collagenase perfusion of the liver, as described previously [15]. Hepatocyte viability was assessed by Trypan Blue exclusion, and initial cell viability in each experiment was more than 85%. 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 carbogen (95% O_2/5% CO_2). Reactions were started by the addition of propyl-paraben (1 mM) dissolved in DMSO (final concentration, < 1%). Corresponding control groups received an equivalent volume of DMSO. In some treated groups, cyclosporin A (5 μM) dissolved in ethanol and trifluoperazine (10 μM) dissolved in Krebs–Henseleit buffer were added into the hepatocyte suspension 30 min before the addition of paraben, and then diazinon (100 μM) dissolved in DMSO was added to the hepatocyte suspension 15 min before the addition of paraben. The concentrations of these modulators used in this study were based on previous studies [6, 16]. Aliquots of the incubation mixture were taken at intervals for the determination of cell death and the concentration of intracellular ATP.

Preparation of Liver Mitochondria

Liver mitochondria were isolated from male F344/DuCrj rats by differential centrifugation in medium containing 0.25 M sucrose, 5 mM Tris–HCl, pH 7.4, and 1 mM EDTA [13]. EDTA was omitted for the final wash and resuspension.

Determination of MPT

Induction of MPT was monitored by the absorbance changes at 540 nm measured with a Cary 5 spectrophotometer (Varian Instrument Ltd.; operated in the double beam mode) equipped with magnetic stirring and temperature control, essentially as described by Petronilli *et al.* [14]. The reaction medium was 0.2 M sucrose, 1 mM KH_2PO_4, 5 mM succinate, 2 μM rotenone, 1 μg/mL of oligomycin, 10 mM Tris–MOPS, pH 7.3, at 25°. The reaction was started by the addition of 0.5 mg protein/mL of mitochondria. Parabens and $CaCl_2$ (50 μM) were added as indicated in Figs. 1 and 2.

Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\Psi$) in hepatocytes was determined with rhodamine 123, a fluorescence probe [17], which selectively distributes into mitochondria with an intact membrane potential and is retained in the mitochondria, as described previously [6]. Rhodamine 123 (1 μM) dissolved in DMSO was added to hepatocyte suspensions 10 min before the exposure to paraben. The results are expressed as percentages of the fluorescence values for the control (untreated) hepatocytes.

Biochemical Assays

ATP in hepatocytes was measured using HPLC, according to the procedure of Jones [18]. Mitochondrial protein was determined with the biuret reaction. Cell death of hepatocytes was assessed by Trypan Blue (0.16%) uptake, according to the method described by Moldéus *et al.* [15]. Plasma membrane blebbing was measured by counting the number of Trypan Blue-excluding cells that had bubble-like protrusions on the cell surface.

Partition Coefficient

The partition coefficients were determined at approximately 25° as follows; 4 μL (final concentration, 50 μM) of a solution of parabens dissolved in DMSO was added to 4 mL of PBS (pH 7.4) saturated with *n*-octanol, and the absorbance was measured, using a spectrophotometer, at a suitable absorption maximum. The solution was added to 4 mL of *n*-octanol, and was extracted by a mechanical shaker for 30 min. After separation (1000 *g* for 20 min) of the *n*-octanol layers by centrifugation, the absorbance of the aqueous layer was measured again. The partition coefficient was calculated as the difference between these two values divided by the residual absorption of the aqueous phase.

Statistical Analysis

Statistically significant differences between several treatment groups were determined by analysis of variance, followed by Dunnett's *t*-test.

RESULTS

Effects of Parabens on MPT

Depletion of cellular ATP and mitochondrial depolarization caused by parabens in isolated rat hepatocytes [6]

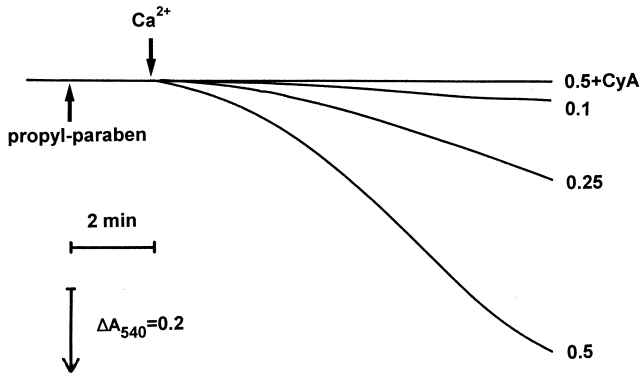

FIG. 1. Concentration-dependent induction of MPT by propyl-paraben. Mitochondria (0.5 mg protein/mL) were preincubated in 2.5 mL of reaction medium, containing 0.2 M sucrose, 1 mM KH_2PO, 5 mM succinate, 2 μM rotenone, 1 μg/mL of oligomycin, and 10 mM Tris–MOPS (pH 7.3) at 25°. Cyclosporin A (CyA; 0.2 μM) was added to the mitochondrial suspension prior to the addition of propyl-paraben. The paraben (0.1 to 0.5 mM) and $CaCl_2$ (50 μM) were added as indicated in the figure, and swelling of mitochondria was determined spectrophotometrically at 540 nm. The traces are typical of three different preparations.

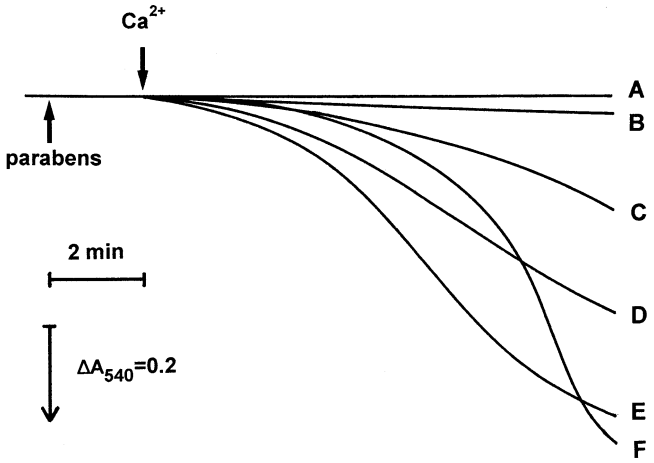

FIG. 2. Induction of MPT by parabens and *p*-hydroxybenzoic acid. The reaction conditions were the same as described for Fig. 1. The concentration of methyl-paraben (trace B), ethyl-paraben (C), propyl-paraben (E), and *p*-hydroxybenzoic acid (D) was 0.5 mM, and that of butyl-paraben (F) was 0.25 mM. Trace A is a non-paraben (control) group. The traces are typical of three different preparations.

suggest that the compounds may induce MPT. When membrane permeability control is inhibited, mitochondria swell owing to the penetration of the solutes. To investigate this proposal, propyl-paraben was added to suspensions of mitochondria, and the swelling of mitochondria via induction of MPT was measured spectrophotometrically (Fig. 1). Incubation of mitochondria with the paraben caused a time- and concentration-dependent MPT. Addition of Ca^{2+} at a concentration of 50 μM to isolated mitochondria without the paraben did not affect MPT during the incubation period. Cyclosporin A (0.2 μM), an inhibitor of MPT via the cyclosporin A-sensitive "mega-pore or mega-channel" [19, 20], or ruthenium red (1 μM; data not shown), which also inhibits MPT by blocking mitochondrial calcium uptake by the uniport pathway [21], completely prevented mitochondrial swelling caused by 0.5 mM propyl-paraben in the presence of Ca^{2+}. These findings indicate that the swelling induced by the paraben represents onset of the MPT.

Figure 2 shows effects of some parabens and *p*-hydroxybenzoic acid on the MPT. Although addition of these parabens to mitochondrial suspensions caused swelling in an alkyl chain-length and time-dependent manner, *p*-hydroxybenzoic acid also exhibited swelling ability, and its potency was greater than that of methyl- or ethyl-paraben. The pre-addition of cyclosporin A (0.2 μM) to mitochondria completely prevented mitochondrial swelling caused by these compounds in the presence of Ca^{2+} (data not shown).

Partition Coefficients of Parabens in Octanol/PBS

A hydrophobic interaction between chemicals and membrane components is a characteristic biological action on the cell and cell membrane [22, 23]. The hydrophobicity of these parabens was therefore determined as the *n*-octanol/PBS partition coefficient (Table 1): the mean values of *p*-hydroxybenzoic acid and methyl-, ethyl-, propyl- and butyl-parabens were 1.1, 21.7, 32.8, 35.7, and 38.4, respectively. The hydrophobicity was increased with elongation of the alkyl side-chains esterified to the carboxyl group of *p*-hydroxybenzoic acid.

Effects of MPT Inhibitors on Paraben-induced Cytotoxicity

Based on the above results, an experiment was performed to determine if inhibitors of MPT could prevent propyl-paraben-induced cytotoxicity (Fig. 3). In a previous study [6], incubation of rat hepatocytes with paraben caused a concentration- and time-dependent cell killing, which was enhanced by the pretreatment of a carboxylesterase inhibitor, diazinon (100 μM). Exposure of hepatocytes to both the paraben and diazinon accelerated cell killing as well as

TABLE 1. Partition coefficients of parabens in *n*-octanol/PBS

Parabens	Partition coefficients*
p-Hydroxybenzoic acid	1.1 ± 0.2
Methyl-paraben	21.7 ± 3.1
Ethyl-paraben	32.8 ± 1.2
Propyl-paraben	35.7 ± 3.5
Butyl-paraben	38.4 ± 2.0

* A solution of parabens (final concentration, 50 μM) dissolved in DMSO was added to 4 mL of PBS (pH 7.4), and the absorbance was measured at a suitable absorption maximum. The solution was extracted by shaking with an equal volume of *n*-octanol saturated with PBS. After separation of the *n*-octanol layers by centrifugation, the absorbance of the aqueous layer was measured again. The partition coefficient was calculated as the difference in these two values divided by the residual absorption of the aqueous medium. Results are the means ± SEM of three experiments.

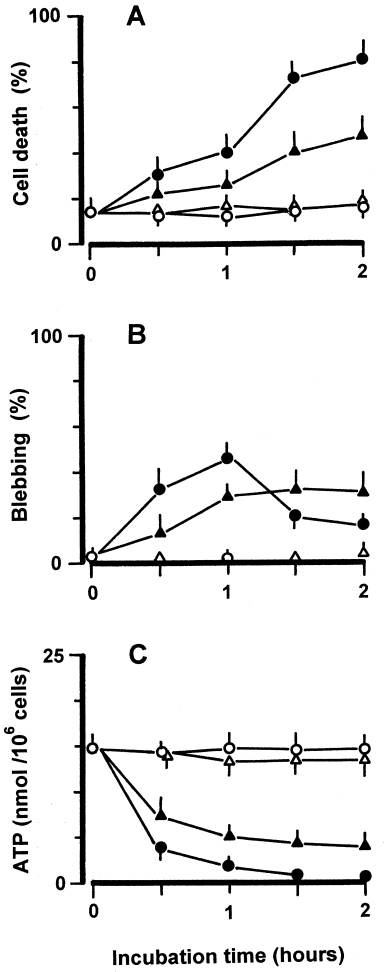

FIG. 3. Effects of cyclosporin A and trifluoperazine, inhibitors of MPT, on cell death (A), cell blebbing (B), and the level of intracellular ATP (C) in isolated rat hepatocytes treated with propyl-paraben. Hepatocytes (10^6 cells/mL) pretreated with an esterase inhibitor, diazinon (100 μM), and/or cyclosporin A (5 μM) plus trifluoperazine (10 μM) were incubated with propyl-paraben (1 mM). Key: (○) untreated; (△) cyclosporin A/trifluoperazine/diazinon-treated; (●) diazinon and propyl-paraben-treated; and (▲) diazinon/cyclosporin A/trifluoperazine/propyl-paraben-treated. Results are the means ± SEM of three different preparations.

the loss of cellular ATP. Trifluoperazine is a potent inhibitor of phospholipase A_2 and an antagonist of calmodulin [16]. The combination of cyclosporin A (5 μM) and trifluoperazine (10 μM) inhibited partially but not completely the paraben-induced cell death accompanied by loss of intracellular ATP during the incubation period. Although the data in Fig. 3 did not show this, loss of mitochondrial membrane potential of hepatocytes treated with propyl-paraben (plus diazinon) was inhibited slightly by both inhibitors after a 90-min incubation; the values of untreated, cyclosporin A/trifluoperazine/diazinon-pretreated, diazinon/propyl-paraben-treated, and cyclosporin A/trifluoperazine/diazinon/propyl-paraben-treated groups were 100, 167, 79, and 66% (% of untreated group; means of two determinations), respectively. Both inhibitors also delayed the onset of plasma membrane blebbing and inhibited an increase in the number of blebbed cells induced by propyl-paraben during the incubation. Diazinon on its own had no effect on any of the parameters measured.

DISCUSSION

The results obtained in this study show that in isolated liver mitochondria propyl-paraben elicited a concentration-dependent induction of mitochondrial swelling dependent upon MPT, which was prevented by pretreatment with an inhibitor of MPT, cyclosporin A (or ruthenium red). Induction of MPT in isolated mitochondria by other parabens tested depended upon the relative elongation of alkyl side-chains in their molecular structures. Because the pretreatment of hepatocytes with cyclosporin A plus trifluoperazine, which are required for a long-lasting inhibition of MPT and act in a synergistic manner [24], partially but not completely prevented diazinon plus propyl-paraben-induced cell death and ATP depletion, it appears that propyl-paraben causes cytotoxicity through mitochondrial dysfunction involving induction of MPT and reduction of mitochondrial membrane potential accompanied by abrupt loss of intracellular ATP.

It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of xenobiotics, is often associated with biological action, which is expressed as structure-toxicity or activity relationship [22, 23]. Although *p*-hydroxybenzoic acid was a potent inducer of MPT (Fig. 2), the compound was less toxic than the shorter-chain esters, methyl- and ethyl-parabens, in isolated rat hepatocytes or in isolated hepatic mitochondria [6]. An analysis of the comparative toxicities of the esters of various parabens shows that their cytotoxicity depends on the relative elongation, or molecular weight, of the alkyl side-chains esterified to the carboxyl group of *p*-hydroxybenzoic acid [6]. As shown in Table 1, the introduction and elongation of these side-chains caused a marked increase, approximately 20- to 35-fold, in the hydrophobicity of the parent compound, *p*-hydroxybenzoic acid. These results imply that the longer-chain esters would readily partition into the interior hydrophobic portion of the plasma membrane, and may pass through cell membranes to reach intracellular target sites such as mitochondria.

In isolated liver mitochondria, many xenobiotics induce the onset of MPT, including oxidant chemicals, heavy metal, and weak acids [10, 21, 25]. The permeability transition pore is voltage dependent, the threshold potential being highly influenced by the redox state of membrane-associated thiols and matrix pyridine nucleotides [14]. It is thought that these redox sensors are acted upon by many inducing agents. However, the cytotoxicity induced by propyl-paraben is independent of protein thiol oxidation and lipid peroxidation in isolated rat hepatocytes [6]. Although the mechanism by which propyl-paraben promotes the onset of MPT remains unclear in this study, Trost and Lemasters [21] have suggested that salicylate (*o*-

hydroxybenzoate) and related carboxylic acids may bind directly to the permeability transition pore, promoting pore opening. In addition, Broekemeier and Pfeiffer [26] have suggested that carboxylic acids having non-polar side-chains induce the MPT accompanied by changing mitochondrial membrane surface potential, thereby decreasing the gating potential of the transition pore. Further work is required to clarify the mechanism of action of parabens.

A previous study indicates that incubation of hepatocytes with parabens results in acute cell death and that the cytotoxicity is associated with ATP depletion through impairment of mitochondrial function related to membrane potential ($\Delta\Psi$) and/or oxidative phosphorylation [6]. A number of studies indicate that mitochondria are a primary target of chemical-induced cell injury and that their dysfunction ultimately leads to cell death. Although the relationship between loss of mitochondrial $\Delta\Psi$ and MPT is complex, it seems that decreased $\Delta\Psi$ is caused by MPT because mitochondrial $\Delta\Psi$ appears to decrease after MPT has begun [8, 11, 27, 28]. MPT is believed to be an important step in cell damage and death in a variety of diseases or pathological states such as xenobiotic poisonings and ischemia/reperfusion, and it can involve both necrosis and apoptosis [9, 29–31]. Broekemeier and Pfeiffer [32] have reported that MPT may be caused by two different or interactive mechanisms, one dependent on phospholipase A_2 activity, which is inhibited by trifluoperazine, and the other on a "pore" in the inner membrane, which is blocked by cyclosporin A. In this study, the evidence that co-treatment of hepatocytes with both inhibitors could prevent propyl-paraben-induced cytotoxicity partially but not completely (Fig. 3) indicates that induction of MPT is an important factor as well as depolarization and/or uncoupling of oxidative phosphorylation in the onset of cell death caused by parabens. The involvement of several independent mechanisms that may interact cannot be ruled out.

Propyl-paraben produced blebbing of the plasma membrane, which is considered to be an early morphological index of chemically induced toxicity and hypoxic damage in hepatocytes [7]. The formation of "blebs" may be causally related to depletion of ATP because the latter is necessary for polymerization of microtubules and microfilaments, which are involved in the interactions between the cytoskeleton and plasma membrane [33]. It has also been suggested that this phenomenon can be caused by either a collapse of the mitochondrial membrane potential ($\Delta\Psi$) or an elevation of cytosolic free Ca^{2+} [33]. The former can result from an impairment of electron transport and uncoupling, both of which were observed in a previous study [6]. Collapse of $\Delta\Psi$ can lead, in turn, to the inability of mitochondria to take up calcium from the cytosol, with a consequent rise in the cytosolic level. A decrease of the cellular ATP level would also affect adversely transport of calcium into the endoplasmic reticulum, which is an energy-requiring process. In this study, protection by both inhibitors from MPT and cell death induced by propyl-paraben was accompanied by a delay in onset of blebbing with intracellular ATP depletion (Fig. 3). A rise in cytosolic free Ca^{2+} induced by parabens has been found by Sone *et al*. [34] in smooth muscle, although in this case it may be related to activation of a calcium channel.

In conclusion, the present study demonstrated that parabens are potent inducers of the mitochondrial cyclosporin A-sensitive permeability transition in isolated mitochondria. The cytotoxicity caused by parabens is characterized by MPT accompanied by mitochondrial depolarization and failure of oxidative phosphorylation with ATP depletion. The insufficient supply of ATP, in turn, limits activities of all energy-requiring reactions and consequently leads to acute cell death.

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