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4-CHLORO-4′-BIPHENYLOL AS AN UNCOUPLER AND AN INHIBITOR OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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Abstract—4-Chloro-4′-biphenylol (4′-OH-4-CB), a metabolite of 4-chlorobiphenyl (4-CB), stimulated state 4 respiration and released oligomycin-inhibited state 3 respiration of rat liver mitochondria with succinate as the respiratory substrate. When glutamate/malate and β-hydroxybutyrate were used as the substrates, however, 4′-OH-4-CB was ineffective on these parameters. This indicates that 4′-OH-4-CB uncouples oxidative phosphorylation with succinate, but not with glutamate/malate and β-hydroxybutyrate. 4′-OH-4-CB severely inhibited 2,4-dinitrophenol (DNP)-stimulated respiration with glutamate/malate (ID_{50}, 25 μM) and β-hydroxybutyrate (ID_{50}, 32 μM) because of the blockade of electron transfer between NADH and CoQ span, masking the uncoupling action of 4′-OH-4-CB. On the other hand, the inhibition of the respiration with succinate was only apparent at high 4′-OH-4-CB concentrations (ID_{50}, 260 μM). 4′-OH-4-CB also inhibited the oxidation of NADH in submitochondrial particles (ID_{50}, 35 μM). State 3 respiration was more intensely inhibited by 4′-OH-4-CB in the presence of either glutamate/malate (ID_{50}, 23 μM) or β-hydroxybutyrate (ID_{50}, 26 μM) than that in the presence of succinate (ID_{50}, 220 μM). Thus, 4′-OH-4-CB acts as both an uncoupler and an inhibitor of oxidative phosphorylation. The overall *in vitro* effect is to prevent ATP synthesis, which may be an important factor in the mechanism underlying the toxicity of 4-CB.

Polychlorinated biphenyls (PCBs) are toxic environmental contaminants that have been detected in various species of wildlife as well as in human tissues [1, 2]. Mammalian metabolism of PCBs is catalyzed primarily by the hepatic microsomal cytochrome P-450 system [3, 4]. In general, lower chlorinated biphenyls are more rapidly metabolized than the highly chlorinated ones. The role of metabolism in the toxicity of PCBs has been only partially elucidated. Some metabolites of PCBs exhibit greater toxicity than the parent compounds. For example, Yamamoto and Yoshimura reported that the monohydroxylated metabolite of 2,4,3′,4′-tetrachlorobiphenyl has a significantly lower LD_{50} in mice than the parent compound [5]. Another example is that both monohydroxylated metabolite and its epoxide precursor were more toxic than the parent compound, 2,5,2′,5′-tetrachlorobiphenyl in a cultured cell system [6].

4-Chlorobiphenyl (4-CB) is a compound whose hepatic metabolism has been well characterized [7, 8]. Incubation of 4-CB with rat liver microsomes gave 4-chloro-4′-biphenylol (4′-OH-4-CB) as the major metabolite [7]. This metabolite is mutagenic in bacterial test system [9]. Other biochemical effects of this compound are unknown, since few studies have been done.

4′-OH-4-CB is a phenol. This compound, therefore, is expected to act as an uncoupler of oxidative phosphorylation in mitochondria. In the present paper, we evaluate the uncoupling effect of 4′-OH-4-CB on rat liver mitochondria. Additionally, we examine the effect of 4′-OH-4-CB on several components of the mitochondrial electron transport chain. The results indicate that 4′-OH-4-CB can induce the uncoupling action with succinate as the respiratory substrate, but do not show the uncoupling ability when glutamate/malate and β-hydroxybutyrate were used as the substrates. In this case, 4′-OH-4-CB acts as an inhibitor of electron transport chain. On the other hand, state 3 respiration with either glutamate/malate or β-hydroxybutyrate is inhibited markedly by 4′-OH-4-CB more than that with succinate.

MATERIALS AND METHODS

Chemicals. 4′-OH-4-CB was purchased from Ultra Scientific (Hope, RI). The stock solution of this compound was prepared in ethanol. ADP, antimycin A, bovine serum albumin, DNP, β-nicotinamide adenine dinucleotide reduced form (NADH), and rotenone were obtained from Sigma (St. Louis, MO). All other reagents were of the highest purity commercially available.

Mitochondrial isolation. Liver mitochondria were isolated from male Wistar rats weighing 200–300 g in a medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), and 0.1 mM EDTA. EDTA was excluded in a final wash and resuspension [10]. Submitochondrial particles were prepared using Branson sonifier (cell disruptor 185) [11]. Protein concentration was determined by the biuret reaction using bovine serum albumin as a standard [12].

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Oxygen consumption. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode in a closed 2 ml glass vessel mixed with a magnetic stirring bar at 25°, with temperature regulated with a thermostatically controlled bath. The respiratory buffer used to measure respiration of mitochondria and submitochondrial particles consisted of 0.2 M sucrose, 20 mM KCl, 5 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). The respiratory substrates were 5 mM glutamate/5 mM malate, 5 mM β-hydroxybutyrate, and 5 mM succinate. State 3 respiration was initiated by the addition of 150 μM ADP, and uncoupled respiration by the addition of 20 μM DNP.

Spectral assays. The redox state of the cytochrome *b* of the respiratory chain was studied at 25° in the respiratory buffer at wavelength pair, 562–575 nm, using a Shimadzu UV-300 dual-wavelength spectrophotometer. The mitochondria were interacted with 4′-OH-4-CB for 3 min, and then the reduction of the cytochrome *b* was initiated by the addition of 5 mM glutamate/5 mM malate.

In all experiments, the control contained the same volume of solvent (ethanol), and the final concentration of the solvent was less than 1% (v/v). This concentration of solvent did not affect the activities assayed.

RESULTS

The effects of 4′-OH-4-CB on mitochondrial state 4 respiration are reported in Fig. 1. With succinate, a FAD-linked substrate, control rat liver mitochondria had substrate-supported oxygen consumption rate of 18.6 natoms oxygen/min/mg protein. Exposure of the mitochondria to 4′-OH-4-CB caused a marked stimulation of state 4 respiration. 4′-OH-4-CB began to stimulate state 4 respiration at 40 μM. As the concentration was increased, oxygen consumption was accelerated, reaching a peak at 150 μM (101.3 natoms oxygen/min/mg protein), after which further increases in concentration resulted in a concentration-dependent inhibition of the stimulated respiration. To examine further the substrate-specificity, glutamate/malate, and β-hydroxybutyrate, NAD^+-linked substrates, were utilized. With glutamate/malate, control mitochondria had substrate-supported oxygen consumption of 9.1 natoms oxygen/min/mg protein. The addition of 4′-OH-4-CB to a suspension of mitochondria caused no stimu-

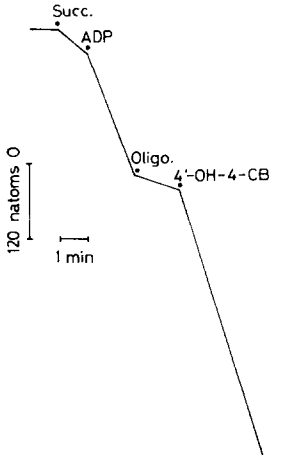

Fig. 2. Release of oligomycin-inhibited state 3 respiration of mitochondria by 4′-OH-4-CB. Rat liver mitochondria (1 mg/ml) were incubated in the respiratory buffer at 25° in a final volume of 2 ml. The additives were: succinate (Succ.), 5 mM; ADP, 1 mM; oligomycin (Oligo.), 1 μg; 4′-OH-4-CB, 150 μM.

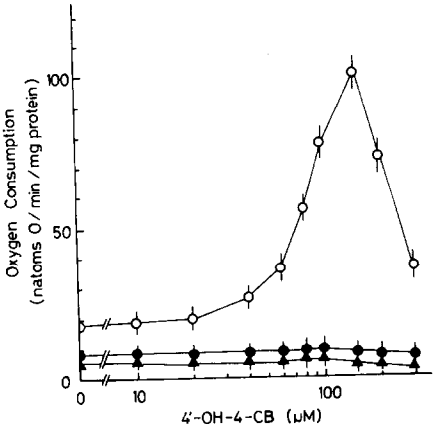

Fig. 1. Effects of 4′-OH-4-CB on state 4 respiration of mitochondria. The respiratory buffer consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). The respiratory substrates were 5 mM succinate (○), 5 mM glutamate/5 mM malate (●), and 5 mM β-hydroxybutyrate (▲). 4′-OH-4-CB was added to state 4 respiring rat liver mitochondria (1 mg/ml). The 4′-OH-4-CB-stimulated respiration was linear to anaerobiosis. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

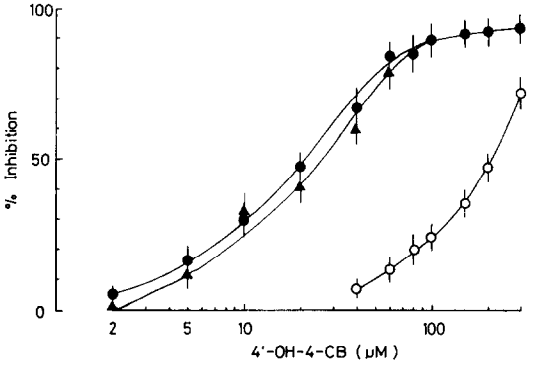

Fig. 3. Effects of 4′-OH-4-CB on state 3 respiration of mitochondria. Rat liver mitochondria (1 mg/ml) were preincubated with 4′-OH-4-CB for 3 min in the respiratory buffer prior to the addition of 5 mM succinate (○), 5 mM glutamate/5 mM malate (●), and 5 mM β-hydroxybutyrate (▲). State 3 respiration was induced by the addition of 150 μM ADP. Values are expressed as percentage inhibition. Each point is a mean ± SD of 3 separate experiments. Control rates of state 3 respiration were 99.9 ± 3.7, 66.5 ± 2.9, and 36.1 ± 1.4 natoms oxygen/min/mg protein for succinate, glutamate/malate, and β-hydroxybutyrate, respectively. Temp. 25°, vol. 2 ml.

lation of state 4 respiration at all concentration ranges tested. When β-hydroxybutyrate was used as the substrate, control mitochondria had substrate-supported oxygen consumption of 7.6 natoms oxygen/min/mg protein. 4'-OH-4-CB did not stimulate state 4 respiration at all concentration ranges tested similar to glutamate/malate as the substrate. As the uncoupling action is reflected by the stimulation of state 4 respiration, the above results indicate that 4'-OH-4-CB acts as an uncoupler with succinate as the substrate, but does not with glutamate/malate, and β-hydroxybutyrate. Namely, the uncoupling action by 4'-OH-4-CB is succinate-specific.

Further experimental evidence that 4'-OH-4-CB acts as an uncoupler with succinate is shown in Fig. 2. The addition of 4'-OH-4-CB released the oligomycin-inhibited state 3 respiration completely with succinate but not with glutamate/malate, and β-hydroxybutyrate (data not shown).

Figure 3 shows the effects of 4'-OH-4-CB on state 3 respiration (phosphorylating respiration). 4'-OH-4-CB inhibited state 3 respiration as low as 2 μM when glutamate/malate was used as the substrate, with 50% inhibition dose (ID_{50}) of 23 μM. Almost identical inhibition was observed with β-hydroxybutyrate as the substrate (ID_{50}, 26 μM). However, with succinate, the inhibition of state 3 respiration was only apparent at high 4'-OH-4-CB concentrations (ID_{50}, 220 μM). Thus, state 3 respiration in the presence of NAD^+-linked substrate was more sensitively inhibited by 4'-OH-4-CB than in the presence of FAD-linked substrate.

Figures 4 and 5 show the effects of 4'-OH-4-CB on DNP-stimulated respiration. These experiments were performed in order to examine whether the electron transport chain is affected by 4'-OH-4-CB. Figure 4 illustrates the representative polarographic traces depicting the order of agent addition. With succinate, DNP-stimulated respiration was not significantly affected by the treatment with 60 μM 4'-OH-4-CB (Fig. 4A and B). When glutamate/malate was used, in contrast, DNP-stimulated respiration was severely inhibited by 60 μM 4'-OH-4-CB (Figs 4C and D). Figure 5 summarizes a concentration-dependence of the effects of 4'-OH-4-CB. With glutamate/malate, 4'-OH-4-CB began to inhibit DNP-stimulated respiration at 2 μM. Above this concentration, the inhibition increased progressively with increasing 4'-OH-4-CB concentration, giving an ID_{50} of 25 μM. With β-hydroxybutyrate, 4'-OH-4-CB also caused a significant inhibition similar to glutamate/malate as the substrate, with ID_{50} of 32 μM. On the other hand, with succinate, the inhibition of the respiration did not appear at relatively low 4'-OH-4-CB concentrations. The respiration was initially inhibited at 40 μM. Above this concentration, as seen with glutamate/malate, and β-hydroxybutyrate, the inhibition increased with

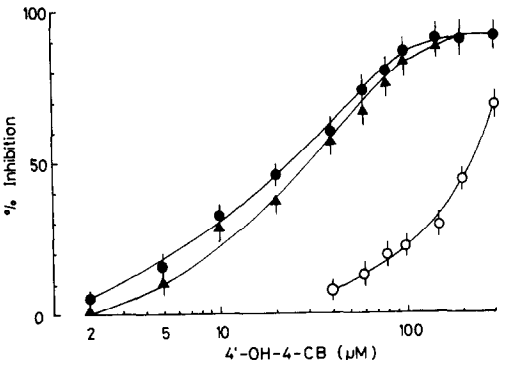

Fig. 5. Concentration-dependence of the effects of 4'-OH-4-CB on DNP-stimulated respiration of mitochondria in the presence of 5 mM succinate (○), 5 mM glutamate/5 mM malate (●), and 5 mM β-hydroxybutyrate (▲). The experimental conditions were as in Fig. 4. Values are expressed as percentage inhibition. Each point is a mean ± SD of 3 separate experiments. Control rates of DNP-stimulated respiration were 114.3 ± 3.6, 69.5 ± 2.9, and 36.7 ± 1.9 natoms oxygen/min/mg protein for succinate, glutamate/malate, and β-hydroxybutyrate, respectively.

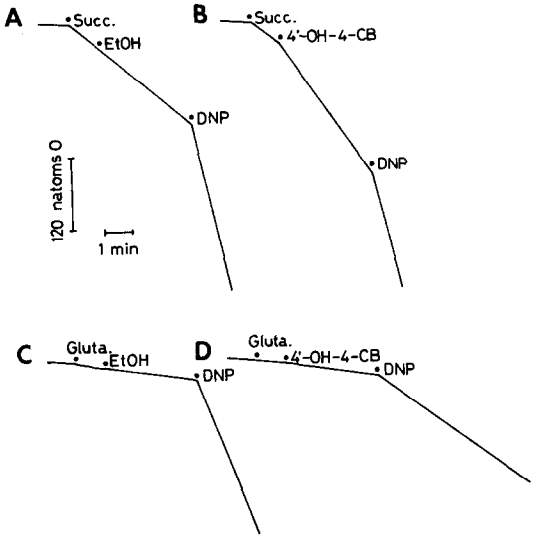

Fig. 4. Representative polarographic traces depicting the effects of 4'-OH-4-CB on DNP-stimulated (uncoupled) respiration of mitochondria. Rat liver mitochondria (1 mg/ml) were preincubated with 60 μM 4'-OH-4-CB for 3 min in the respiratory buffer either with 5 mM succinate (Succ., A and B) or with 5 mM glutamate/5 mM malate (Gluta., C and D), then uncoupled respiration was initiated by the addition of 20 μM DNP. Temp. 25°, vol. 2 ml.

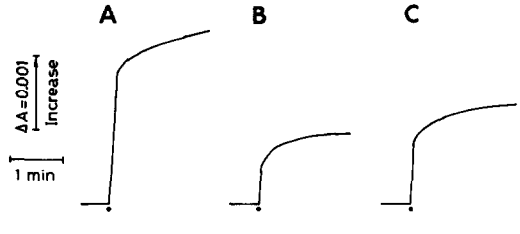

Fig. 6. Effects of 4'-OH-4-CB on the reduction of cytochrome *b* by glutamate/malate in mitochondria. Rat liver mitochondria (2 mg/ml) were interacted either with rotenone or with 4'-OH-4-CB for 3 min, then the reduction of cytochrome *b* was initiated by the addition (●) of 5 mM glutamate/5 mM malate: (A) control; (B) rotenone, 2 μM; (C) 4'-OH-4-CB, 60 nmol/mg protein. Temp. 25°, vol. 2.5 ml.

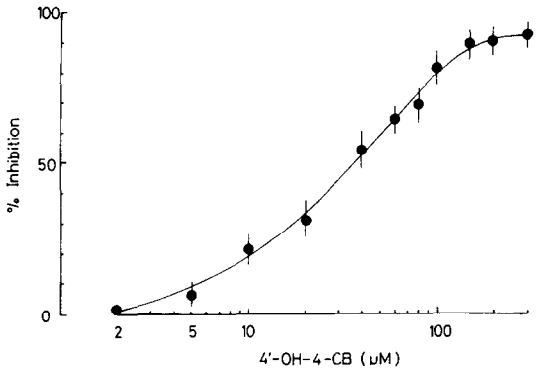

Fig. 7. Effects of 4'-OH-4-CB on respiration of submitochondrial particles. Submitochondrial particles (1 mg/ml) were preincubated with 4'-OH-4-CB for 3 min in the respiratory buffer, then the respiration was initiated by the addition of 0.8 mM NADH. Values are expressed as percentage inhibition. Each point is a mean ± SD of 3 separate experiments. Control rate of respiration was 201.4 ± 5.6 natoms oxygen/min/mg protein. Temp. 25°, vol. 2 ml.

increasing 4'-OH-4-CB concentration (ID_{50}, 260 μM). The results of Figs 4 and 5 indicate that the electron transport from NAD^+-linked substrate is much more sensitive to inhibition by 4'-OH-4-CB than that from FAD-linked substrate.

To further localize the site of inhibition in the electron transfer by relatively low concentration of 4'-OH-4-CB (i.e. concentration which does not significantly affect the succinate oxidation), the redox state of cytochrome *b* was examined. Figure 6A shows the control reduction of cytochrome *b* by glutamate/malate. Preincubation of mitochondria with rotenone, a well known inhibitor of the NADH-CoQ span, reduced the reduction of cytochrome *b* to 30% of control (Fig. 6B). Likewise, preincubation of mitochondria with 4'-OH-4-CB (60 nmol/mg protein) suppressed cytochrome *b* reduction by 50% of control (Fig. 6C), indicating its possible site of action to be on the substrate side of cytochrome *b*.

The actions of 4'-OH-4-CB were also studied by examining its effects on the oxidation of NADH by submitochondrial particles. As shown in Fig. 7, 4'-OH-4-CB induced significant inhibition of the oxidation of NADH (ID_{50}, 35 μM); the extent of which was almost similar to that seen in the NAD^+-linked substrate oxidation with intact mitochondria (Fig. 5).

DISCUSSION

Many phenols have been shown to uncouple oxidative phosphorylation in mitochondria [13], and our study examines whether 4'-OH-4-CB, which is also phenol, shows an uncoupling action. The results of the current study demonstrate that 4'-OH-4-CB has the ability to uncouple oxidative phosphorylation with succinate as the substrate. This was evidenced by the findings that 4'-OH-4-CB: (a) stimulated state 4 respiration with succinate (Fig. 1); (b) released oligomycin-inhibited state 3 respiration completely (Fig. 2). On the other hand, with glutamate/malate and β-hydroxybutyrate, 4'-OH-4-CB neither stimulated state 4 respiration nor released oligomycin-inhibited state 3 respiration, indicating that 4'-OH-4-CB does not exert the uncoupling action. This is in contrast to a typical phenolic uncoupler, DNP, which shows the uncoupling action both with succinate and with glutamate/malate and β-hydroxybutyrate.

Figure 8 shows an abbreviated representation of the enzyme complexes of the electron transport system in mitochondria including the apparent sites of action of inhibitors. 4'-OH-4-CB, at low concentrations, severely inhibited the NAD^+-linked oxidation of glutamate/malate and β-hydroxybutyrate in intact mitochondria (Fig. 5). This compound also caused almost identical inhibition of the oxidation of NADH by submitochondrial particles (Fig. 7); the experiment using submitochondrial particles can exclude the influence of both substrate transport and matrix reaction. These facts indicate that substrate transport system is not affected by 4'-OH-4-CB. 4'-OH-4-CB showed the ability to prevent the reduction of cytochrome *b* with glutamate/malate (Fig. 6),

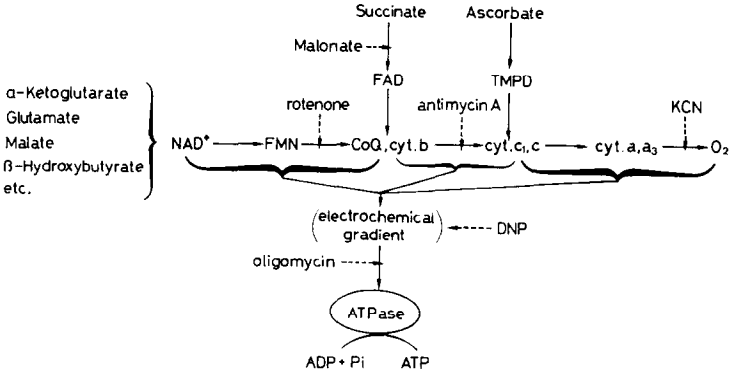

Fig. 8. Scheme of the energy-transducing system linked to the respiratory chain of mitochondria. Broken arrows indicate the apparent sites of action of inhibitors. Solid arrows indicate paths of electron flow. cyt., cytochrome; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine.

suggesting that 4'-OH-4-CB interferes with the components before cytochrome *b* of the electron transfer system (Fig. 8), probably TCA-cycle dehydrogenase or NADH-CoQ segment. However, the following evidence rules out TCA-cycle dehydrogenase: mitochondrial oxygen consumption with either glutamate/malate or β-hydroxybutyrate as the substrate was inhibited to a similar extent by 4'-OH-4-CB (Fig. 5); the oxidation of the former substrate occurs via TCA-cycle dehydrogenase while that of the latter does not. Furthermore, 4'-OH-4-CB did not inhibit the reduction of NAD^+ in mitochondria by the addition of glutamate/malate (data not shown, measured with the dual-wavelength spectrophotometer at wavelength pair, 340–375 nm), from which electrons enter the respiratory chain at the level of NAD^+. This is further evidence that 4'-OH-4-CB does not affect the TCA-cycle dehydrogenase. Therefore, the suppression of the reduction of cytochrome *b* by 4'-OH-4-CB is attributed to the inhibition of the NADH-CoQ segment of the electron transport chain, at a locus that may be either identical to or close to the rotenone sensitive site. When succinate was used as the substrate, 4'-OH-4-CB only inhibited the electron transfer at concentrations more than 40 μM. The maximum inhibition observed was 69% at 300 μM. This indicates that at high concentrations, 4'-OH-4-CB inhibits either succinate dehydrogenase or the electron transport chain common to both NAD^+- and FAD-linked substrates (*b*–c_1 complex, and cytochrome *c* oxidase) or both. The inability of 4'-OH-4-CB to show uncoupling action with either glutamate/malate or β-hydroxybutyrate is due to greater inhibition of the electron transfer with these substrates than with succinate; the uncoupling action of 4'-OH-4-CB is masked because of the increased inhibition of the electron transfer.

The ability of 4'-OH-4-CB to uncouple oxidative phosphorylation with succinate is weak when compared to DNP which shows the full uncoupling at 10–20 μM. This may be attributed to lesser acidity of 4'-OH-4-CB (pK_a, 9.6 [14]) compared to that of DNP (pK_a, 4.0 [13]). Among the metabolites of PCBs, the greater degree of chlorination on the phenyl ring bearing the hydroxyl group generally leads to greater acidity [14]. Therefore, it may be expected that there exist potent uncouplers within the phenolic metabolites of PCBs with the greater degree of chlorination, which is interesting for the toxic mechanism of PCBs.

Finally, toxicological significance of the damage to mitochondria by 4'-OH-4-CB in evaluating the toxicity of 4-CB is discussed. Low concentrations of 4'-OH-4-CB caused a profound defect in the electron transport chain at the NADH-CoQ segment, impairing the utilization of NAD^+-linked substrates, which results in a pronounced reduction of ATP synthesis. In fact, with glutamate/malate and β-hydroxybutyrate, 4'-OH-4-CB severely inhibited state 3 respiration (Fig. 3). At high concentrations, it uncoupled oxidation from phosphorylation with succinate, also preventing ATP synthesis. Regarding the toxic phenomena by an uncoupling agent, George *et al.* reported alterations of morphology of cultured liver cells by DNP; it caused significant blebs on the cell surface associated with a loss of microvilli [15]. Mitochondria were swollen with a pale intramitochondrial matrix which was totally devoid of dark staining deposits [15]. DNP also decreased ATP levels of the cells [15]. Similarly, the same damage on the cells may be expected by 4'-OH-4-CB as an uncoupler. Thus, 4'-OH-4-CB acts as both an inhibitor and an uncoupler of oxidative phosphorylation. The overall *in vitro* effect of 4'-OH-4-CB on mitochondria is to prevent ATP synthesis, which leads to diminished activity of cellular energy-dependent processes. Although the effects of 4-CB on mitochondria remains to be elucidated, its metabolite, 4'-OH-4-CB, acts as a mitochondrial toxin, which may be an important factor in mechanisms underlying the toxicity of 4-CB.

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