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The Natural Antioxidant Otobaphenol Delays the Permeability Transition of Mitochondria and Induces Their Aggregation

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ABSTRACT

The lignan otobaphenol, (8*R*,8′*R*,7*R*)-4′-hydroxy-5′-methoxy-3,4-methylenedioxy-2′,7,8,8′-neolignan, extracted from *Virola Aff. Pavonis* leaves, completely inhibits at a concentration of 2.5 μ*M* the Fe^{3+}-ascorbate-induced lipoperoxidation of rat liver mitochondria that was determined by oxygen consumption and accumulation of thiobarbituric acid-reactive species. At 25 μ*M*, it delays the mitochondrial permeability transition induced by *tert*-butyl hydroperoxide or Ca^{2+}, substantially inhibits the state 3 respiration, does not affect the state 4 respiration and the ADP/O ratio (with succinate), diminishes the rate of Ca^{2+} uptake by mitochondria, and delays the ruthenium red-insensitive uncoupler-induced release of the loaded Ca^{2+}. Dose-dependent delaying of the calcium-induced swelling of mitochondria in the presence of otobaphenol nonlinearly correlates with its 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity. At 75 μ*M* and higher, this lignan causes mitochondrial aggregation and is able to aggregate itself, without mitochondria. The formed aggregates of otobaphenol do not cause an aggregation of subsequently added mitochondria. Thus, otobaphenol seems to be a promising target to prevent the oxidative stress death of cells. *Antioxid. Redox Signal.* 5, 281–290.

INTRODUCTION

PERMEABILITY TRANSITION of the inner mitochondrial membrane (IMM) is an essential step in cell necrosis and some types of apoptosis (7, 19, 21, 28, 30, 37). Prooxidants and oxidative stress significantly increase the probability of the IMM permeability transition (12, 17, 21, 22). Natural antioxidants seem to be of great interest to protect mitochondria against damage caused by reactive oxygen species, because free radials participate in a synergistic process of the permeability transition pore opening (3, 12, 21).

Although a great body of experimental data on various factors influencing the IMM permeability has been obtained (3, 12, 18, 21, 22), the structural basis and molecular mechanisms of the permeability transition pore opening have not yet been fully elucidated (3, 12, 21). Among the factors that modulate the IMM permeability, one can count the following: oxidation of critical vicinal thiols in the IMM (2, 3) or their reactions with the bifunctional reagents (9, 29), the dithiol–disulfide exchange and the IMM protein cross-linkage (2–4, 21), the redox state of endogenous pyridine nucleotides (3, 11, 21), nitric oxide (NO) and peroxynitrite generation (16, 33, 38), as well as the membrane potential value, matrix pH, Ca^{2+}, and ADP concentrations (3, 12, 28, 39). It has been suggested that free radicals are central promoting elements of the IMM permeabilization *in vivo* (8, 12, 19–21).

Various antioxidants have been shown to delay the IMM permeability transition (17, 19, 21). Certain medicinal plants, *e.g.,* the plants of the family Myristicaceae, are rich in the natural antioxidants (13). Oil-soluble resins extracted from some of these plants contain a relatively high quantity of phenolic substances and possess an antioxidant activity even higher than that of butylated hydroxytoluene (BHT) (13), one of the best commercially available antioxidants. The natural compound otobaphenol may be obtained from the leaves of *Virola Aff. Pavonis* (Myristicaceae), and its spectroscopic

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characteristics and structural formula have been described earlier (27). However, its antioxidant and biological activities have not yet been studied to the best of our knowledge. In particular, its capability to inhibit the free radical-induced lipoperoxidation of mitochondrial membranes and the oxidative stress-induced permeability transition of the IMM, as well as its influence on respiration and oxidative phosphorylation of mitochondria, have not yet been studied.

In this work, we show that otobaphenol inhibits the Fe^{3+}-ascorbate-induced lipoperoxidation of mitochondrial membranes, does not uncouple the oxidative phosphorylation and respiration, but substantially decreases the state 3 respiration of rat liver mitochondria. It also partially inhibits the uptake and the ruthenium red-insensitive release of Ca^{2+} and delays the IMM permeability transition induced by *tert*-butyl hydroperoxide (*t*-BOOH) or by loading mitochondria with Ca^{2+}. On the other hand, at relatively high concentration, otobaphenol causes mitochondrial aggregation and also aggregates itself, even in the absence of mitochondria. The aggregates of otobaphenol do not induce aggregation of subsequently added mitochondria, but even protected them against oxidative-stress damages.

MATERIALS AND METHODS

Sucrose, D-mannitol, HEPES, Trizma base, EGTA, free of fatty acids bovine serum albumin (fraction V), cyclosporin A, rotenone, L-ascorbic acid, glutamic acid, malic acid, succinic acid, 2-thiobarbituric acid (TBA), phosphoric acid, ADP, BHT, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), *t*-BOOH, $CaCl_2$, ruthenium red, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals were of analytical grade. (+)-Otobaphenol, (8*R*,8′*R*,7*R*)-4′-hydroxy-5′-methoxy-3,4-methylenedioxy-2′,7,8,8′-neolignan, was extracted from the air-dried leaves of *Virola Aff. Pavonis,* isolated, and purified as described (27). Stock solution of otobaphenol in DMSO was prepared using the white powder of the purified substance and stored at 0–4°C.

Liver mitochondria from male rats, starved for 12–14 h, were isolated by the standard procedure of differential centrifugation using medium containing 210 m*M* mannitol, 70 m*M* sucrose, 1 m*M* EGTA-Tris, 5 m*M* HEPES-Tris, pH 7.4. Mitochondria were washed and finely suspended in medium containing 210 m*M* mannitol, 70 m*M* sucrose, 0.02 m*M* EGTA-Tris, 5 m*M* HEPES-Tris, pH 7.4, and 0.3 mg/ml free of fatty acids bovine serum albumin. To study the antioxidant properties of otobaphenol, mitochondria were washed twice in 125 m*M* Tris-HCl, pH 7.4, to exclude sucrose, which reacts with TBA and thus may affect the results of TBA-reactive species measuring. The content of mitochondrial protein was determined by the biuret method using bovine serum albumin as the standard.

Respiration and oxidative phosphorylation of mitochondria were determined polarographically using the Cole–Parmer Oxygen Meter connected to the Linseis L250-E recorder (U.S.A.) as described (25). Mitochondria (1.0 mg of protein/ml) and 2.5 μ*M* rotenone were added to the incubation medium containing 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES-Tris buffer, pH 7.4 (MSH medium), which was supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. The cumulative contamination of this medium with Ca^{2+} was 14 μ*M* determined by the method of atomic absorption spectrophotometry. After registration of the state 4 respiration for 2 min, 250 μ*M* ADP was added. After phosphorylation of the added ADP was completed, 0.1 m*M* dinitrophenol was added to uncouple the respiration and oxidative phosphorylation. To study the influence of otobaphenol on mitochondrial respiration in comparison with BHT, these compounds were added at a concentration of 25 μ*M* just after mitochondria and rotenone. The initial concentration of oxygen in the medium was considered 200 nmol/ml at 37°C, according to the atmospheric pressure in Medellin.

Antioxidant activity of otobaphenol was assessed by determining the inhibition of the oxygen consumption rate under mitochondrial lipoperoxidation induced by Fe^{3+}-ascorbate at 37°C. Mitochondria (1 mg of protein/ml), washed in 125 m*M* Tris-HCl, pH 7.4, were added together with 2.5 μ*M* rotenone to the buffer of 125 m*M* Tris-HCl, pH 7.4, supplemented with 1 m*M* KCN and 0.5 m*M* ascorbate-Tris. The lipoperoxidation was initiated by 10 μ*M* $FeCl_3$ added after a 1-min preincubation of mitochondria. Except for control, otobaphenol or BHT was added together with mitochondria and rotenone (1 min before $FeCl_3$) at the concentrations indicated in the figure legends. Malondialdehyde and other TBA-reactive species, accumulated for 5- and 10-min incubations, were determined as described previously (6). In another set of experiments, 2.5 μ*M* otobaphenol or 2.5 μ*M* BHT was added 2–3 min after $FeCl_3$, when the rate of oxygen consumption in the lipoperoxidation process was maximal (Fig. 1, traces f and g, respectively).

Free radical DPPH was used to evaluate the free radical scavenging activity of otobaphenol in comparison with the positive control BHT, as described previously (34), with some modifications. Methanolic media were used, to which very small volumes of otobaphenol or BHT solutions in DMSO were added to final concentrations of 0, 5, 15 and 25 μ*M*. These samples, which also contained 30 μ*M* DPPH, were intensively vortexed and incubated at room temperature for 20 min. A decrease of absorbance at 517 nm (A_{517}) was measured every 5 min.

Light scattering by mitochondrial suspension was monitored by the optical density at 540 nm (A_{540}) using an SP-850 spectrophotometer (U.S.A.), equipped with a magnetic stirrer in a thermostatic chamber at 37°C. Mitochondria (0.5 mg of protein/ml), 2.5 μ*M* rotenone, and 1 min later otobaphenol or BHT were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. The absorbance at 540 nm (A_{540}) was recorded with the Linseis recorder connected to the spectrophotometer. The images of the aggregates of otobaphenol (in the medium without mitochondria) or mitochondria-otobaphenol, both formed at 250 μ*M* otobaphenol during 18 min of incubation, were captured by the computerized CCD camera installed on a light microscope (Axiolab-Zeiss, Germany). A sample of 20 μl of mitochondrial suspension was placed on a stage and covered with the cover glass. Mitochondrial swelling in the same incubation medium was induced by 0.3 m*M* *t*-BOOH or 30 μ*M* Ca^{2+} added 1 min after 25 μ*M* otobaphenol or 25 μ*M* BHT.

Redox status of mitochondrial pyridine nucleotides was determined by registering the NAD(P)H fluorescence using an Aminco–Bowman luminescence spectrometer at 37°C as described in our work (24), with excitation at 340 nm and emission at 450 nm. Mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. The oxidation of endogenous NAD(P)H was induced by 30 μ*M* Ca^{2+} or 0.3 m*M* *t*-BOOH in the absence or presence of 25 μ*M* otobaphenol or BHT added 1 min before these inducers.

Mitochondrial Ca^{2+} uptake and Ca^{2+} release were registered by chlorotetracycline fluorescence (26) using an Aminco–Bowman luminescence spectrometer with the modified cuvette holder (24) at excitation and emission wavelengths of 405 nm and 540 nm, respectively. Mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone were added to the MSH medium supplemented with 10 m*M* acetate-Tris, 5 m*M* succinate-Tris, and 2.0 μ*M* chlorotetracycline at 37°C. In some experiments, 1 μ*M* cyclosporin A was also added to prevent the IMM permeability transition. After preincubation for 1.5 min, 100 μ*M* Ca^{2+} was added. The release of the loaded Ca^{2+} was induced by 10 μ*M* ruthenium red and/or 1 μ*M* FCCP. Except for control, 25 μ*M* otobaphenol or BHT was added just after mitochondria.

The trans-IMM electrical potential was monitored by safranin O fluorescence as described earlier using an Aminco–Bowman luminescence spectrometer (25). Mitochondria (0.5 mg of protein/ml), 2.5 μ*M* rotenone, and 1 μ*M* cyclosporin A were added to the MSH medium supplemented with 5 m*M* phosphate-Tris, 5 m*M* succinate-Tris, and 10 μ*M* safranin O at 37°C. After a 1.5-min preincubation with 25 μ*M* otobaphenol or BHT, 100 μ*M* Ca^{2+} was added, causing the trans-IMM potential drop for the time period needed to take up the added Ca^{2+}. Finally, mitochondria were deenergized by 1.0 μ*M* FCCP.

RESULTS

Antioxidant activity of otobaphenol was assessed using the model of Fe^{3+}-ascorbate-induced lipoperoxidation of rat liver mitochondria. The incubation medium contained 1 m*M* KCN to inhibit the oxygen consumption via cytochrome *c* oxidase. Otobaphenol (2.5 μ*M*) added simultaneously with mitochondria completely inhibited the rate of oxygen consumption related to the lipoperoxidation process (Fig. 1, trace c). At a concentration of 1.25 μ*M,* only half-maximal inhibition was observed (Fig. 1, trace b). Antioxidant activity of 2.5 μ*M* otobaphenol was close to that observed with 1.25 μ*M* BHT (Fig. 1, traces c and d, respectively). The accumulation of TBA-reactive species under these conditions (Table 1) was in a good agreement with the oxygen consumption data (Fig. 1, traces a–d).

The addition of antioxidants to the incubation medium when the lipoperoxidation process was maximally activated by 10 μ*M* $FeCl_3$, allowed a comparison of the kinetics of inhibition by otobaphenol and by BHT. The data show that the inhibition by 2.5 μ*M* otobaphenol of the highly activated lipoperoxidation was developed much more slowly than that by 2.5 μ*M* BHT (Fig. 1, traces f and g, respectively). But after

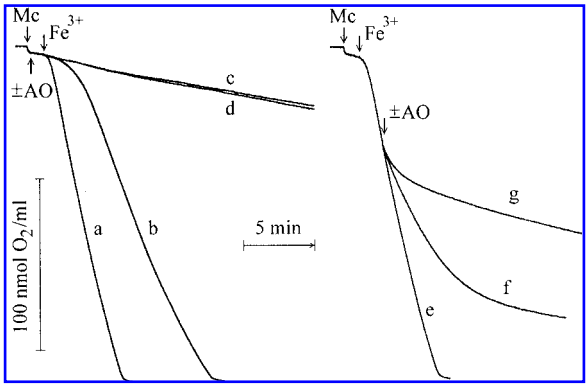

FIG. 1. Cyanide-resistant oxygen consumption under rat liver mitochondria lipoperoxidation induced by Fe^{3+}-ascorbate. Mitochondria (Mc) (1 mg of protein/ml) and 2.5 μ*M* rotenone were added to the buffer of 125 m*M* Tris-HCl, pH 7.4, supplemented with 1 m*M* KCN, 0.5 m*M* ascorbate-Tris, and the following: Fe^{3+}, 10 μ*M* $FeCl_3$; AO, 1.25 μ*M* otobaphenol (b) or 2.5 μ*M* otobaphenol (c, f); 1.25 μ*M* BHT (d) or 2.5 μ*M* BHT (g). Traces a and e represent control.

5–7 min of incubation in the presence of these antioxidants, almost equal rates of the strongly inhibited lipoperoxidation were established.

The DPPH free radical scavenging activity of otobaphenol in methanol (Fig. 2A) was significantly higher than that of BHT (Fig. 2B). The rate of discoloration of DPPH, resulting from its reduction by antioxidants, increased by the antioxidant in a dose-dependent manner. Interestingly, the DPPH scavenging by these antioxidants in the DMSO medium, rather than methanol, was extremely slow, and intermediate results may be observed in mixtures of DMSO/methanol (data not shown).

A significant delay of the spontaneous swelling of mitochondria incubated at 37°C in the MSH medium with succinate and inorganic phosphate was observed at 25 and 50 μ*M* otobaphenol (Fig. 3, traces b and c, respectively). On the other hand, almost immediate decrease in A_{540} occurred when otobaphenol was added 75, 125, or 250 μ*M* (Fig. 3, traces d, e, and f, respectively); this diminishing in the optical density of

TABLE 1. INFLUENCE OF OTOBAPHENOL AND BHT ON ACCUMULATION OF TBA-REACTIVE SPECIES

	TBA-reactive species, A_{535}	
Experimental conditions	*5 min*	*10 min*
Control	0.400 ± 0.018	0.708 ± 0.036
1.25 μ*M* otobaphenol	0.109 ± 0.023	0.337 ± 0.031
2.50 μ*M* otobaphenol	0.008 ± 0.002	0.020 ± 0.005
1.25 μ*M* BHT	0.012 ± 0.005	0.029 ± 0.012

The accumulation of TBA-reactive species was determined following Fe^{3+}-ascorbate-induced lipoperoxidation of rat liver mitochondria (125 m*M* Tris-HCl, pH 7.4, 1 m*M* KCN, 0.5 m*M* ascorbate-Tris; 1 mg of mitochondrial protein/ml). Lipoperoxidation was initiated by 10 μ*M* $FeCl_3$ added after a 1-min preincubation of mitochondria ($n = 4$).

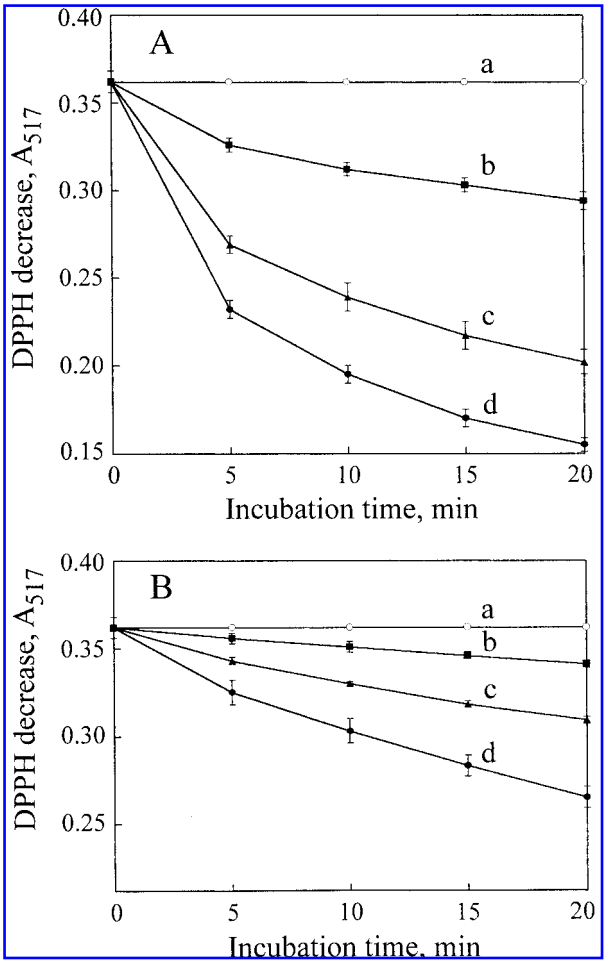

FIG. 2. DPPH free radical scavenging activity of otobaphenol (A) and BHT (B). Antioxidants were added to the methanolic media with 30 μ*M* DPPH at 5 μ*M* (b), 15 μ*M* (c), and 25 μ*M* (d). Curve a represents control. Decrease of the absorbance at 517 nm (A_{517}) was measured during incubation for 20 min.

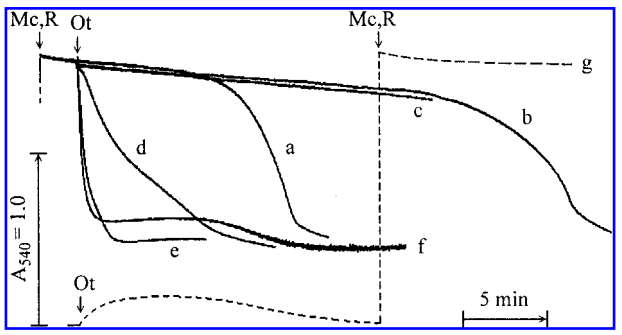

FIG. 3. Optical density changes (A_{540}) of rat liver mitochondria suspension influenced by otobaphenol. Mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone (Mc,R) were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. After a 2-min preincubation of mitochondria, otobaphenol (Ot) was added at the following concentrations: 25 μ*M* (b); 50 μ*M* (c); 75 μ*M* (d); 125 μ*M* (e); 250 μ*M* (f); 250 μ*M* otobaphenol (g) was added to the medium 18 min before the addition of mitochondria.

mitochondrial suspension was insensitive to cyclosporin A (data not shown), and the traces of A_{540} changes had a biphasic character (Fig. 3, traces d–f). The second phase was accompanied with an increasing noise (Fig. 3, trace f) and with the formation of big aggregates, which were visually detected and observed by light microscopy (Fig. 4B). At 250 μ*M* concentration, for example, otobaphenol also aggregated itself in the medium without mitochondria (Fig. 3, trace g) and was accompanied with an increase and a subsequent decrease of

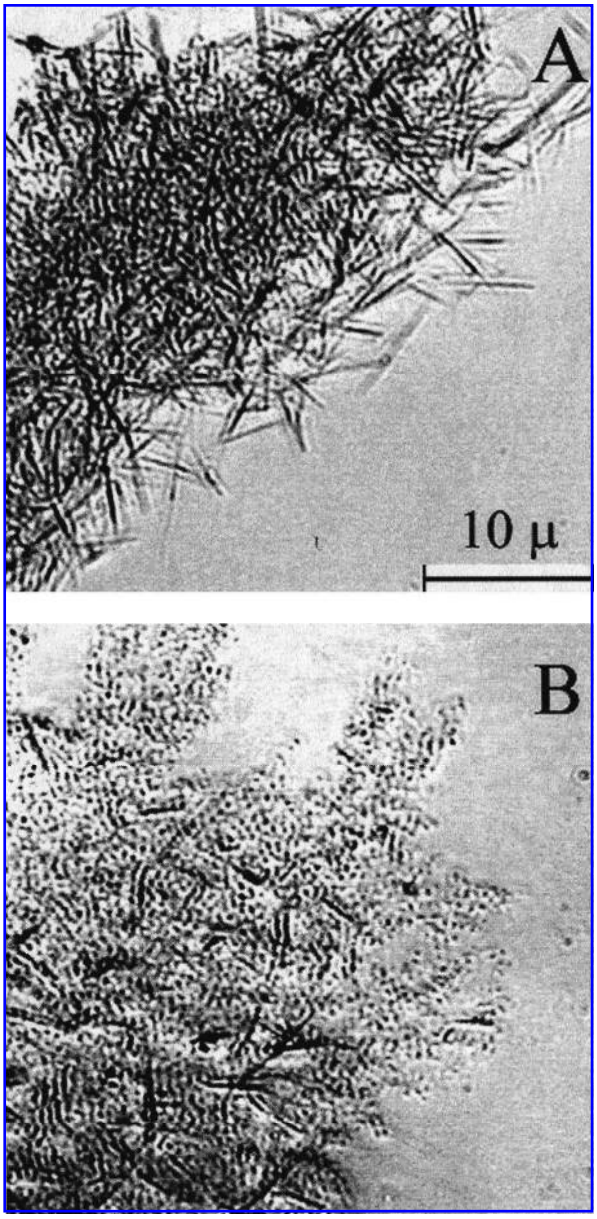

FIG. 4. Optical microscopy images of the structures formed by otobaphenol or mitochondria-otobaphenol. Otobaphenol was added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris without (A) or with (B) rat liver mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone. The images were captured with the CCD camera after an 18-min incubation according to the traces g and f, respectively, in Fig. 3.

A_{540} (Fig. 4A), both resulting from the growth of the aggregates. Mitochondria added after these stages did not aggregate (Fig. 3, trace g) and were even somewhat protected against oxidative stress-induced permeability transition (data not shown).

Mitochondrial swelling was significantly accelerated by addition of 0.3 m*M* *t*-BOOH (Fig. 5, trace a) or 30 μ*M* Ca^{2+} (Fig. 5, trace d). In both these cases, 25 μ*M* otobaphenol significantly delayed the permeability transition (Fig. 5, traces b and e), although the protective effect of BHT was higher (Fig. 5, traces c and f). Antioxidants also influenced the biphasic oxidation of mitochondrial NAD(P)H induced by *t*-BOOH (Fig. 6A, trace a). The second phase results from the swelling of mitochondria and their outer membrane rupture, and may be prevented by cyclosporin A (24). Otobaphenol and BHT had little effect on the first phase, but significantly displaced the second phase (Fig. 6A, traces b and c, respectively) in accordance with delaying of the mitochondrial swelling (Fig. 5, traces b and c, respectively). Both otobaphenol and BHT also inhibited the Ca^{2+}-induced oxidation of the total pool of mitochondrial NAD(P)H (Fig. 6B, traces b and c, respectively). One part of the completely oxidized NAD(P)H pool was recovered by glutamate-malate in the presence of cyanide (Fig. 6), whereas cyanide alone had little effect, similar to that observed earlier (24).

It has been demonstrated that BHT does not influence the mitochondrial permeability transition at concentrations that are sufficient to inhibit the mitochondrial lipoperoxidation (17 and references therein). As one of the possibilities, this protective action of BHT was explained by its allosteric effect on the permeability transition pore (17, 36). With respect to otobaphenol, we also observed that it completely inhibits the Fe^{3+}-ascorbate-induced lipoperoxidation of mitochondria at a concentration of 2.5 μ*M* (Fig. 1, traces c and f, and Table 1), but even at 5.0 μ*M* it shows a little influence on the mitochondrial swelling induced by calcium (Fig. 7, *x* axis). On the other hand, the delay of the mitochondrial swelling was in nonlinear relation to the DPPH scavenging activity of otobaphenol (Fig. 7). At higher DPPH scavenging, *i.e.,* at increased concentration of otobaphenol, a higher delay of the mitochondrial swelling induced by Ca^{2+} was observed.

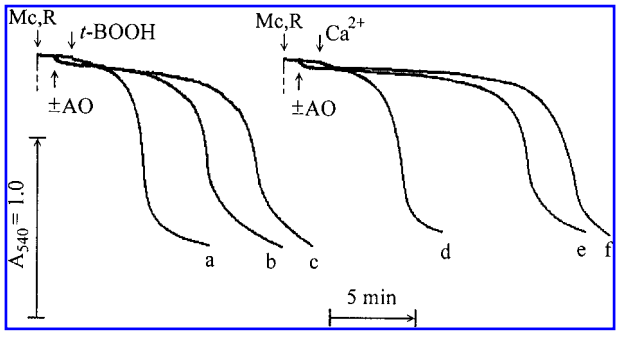

FIG. 5. Influence of otobaphenol and BHT on the rat liver mitochondria swelling induced by *t*-BOOH or Ca^{2+}. Mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone (Mc,R) were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris; *t*-BOOH, 0.3 m*M* *t*-BOOH; Ca^{2+}, 30 μ*M* Ca^{2+}; AO, 25 μ*M* otobaphenol (b, e) or 25 μ*M* BHT (c, f). Traces a and d represent control.

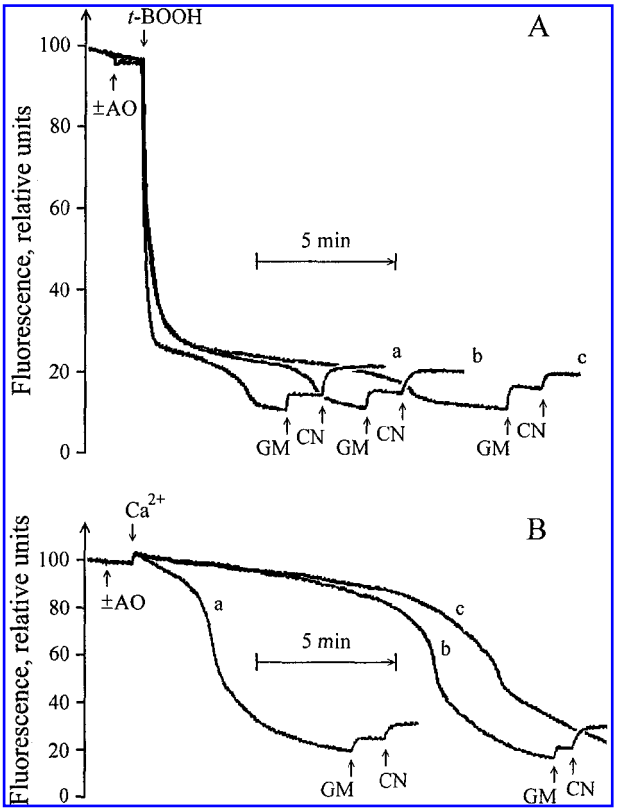

FIG. 6. Influence of otobaphenol and BHT on the fluorescence intensity changes of mitochondrial pyridine nucleotides induced by *t*-BOOH (A) or Ca^{2+} (B). Rat liver mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. After a 1-min preincubation of mitochondria, 25 μ*M* otobaphenol (b) or 25 μ*M* BHT (c) was added (AO); *t*-BOOH, 0.3 m*M* *t*-BOOH; Ca^{2+}, 30 μ*M* Ca^{2+}; GM, 4 m*M* glutamate-Tris plus 1 m*M* malate-Tris; CN, 1 m*M* KCN. Trace a represents control.

Mitochondrial permeabilization in the presence of Ca^{2+} results from accumulation of this ion in the mitochondrial matrix (3, 12, 28). It is known that Ca^{2+} overload activates the mitochondrial NO synthase (15, 16, 38). In the mitochondrial matrix, NO and superoxide anion free radicals produce peroxynitrite (35), which has been shown to induce the Ca^{2+} efflux from mitochondria and to cause the IMM permeability transition (33). The data in Fig. 8 show that neither antioxidant prevented the mitochondrial Ca^{2+} uptake, but only partially inhibited it. Essentially biphasic changes of the chlorotetracycline fluorescence under the Ca^{2+} uptake were observed, which were different for the presence of otobaphenol and BHT (Fig. 8A, traces b and c, respectively). The release of Ca^{2+} from the mitochondrial matrix (Fig. 8A, traces a–c) seems to be involved in the permeability transition pore opening, resulting in subsequent swelling of mitochondria (Fig. 5, traces d–f). In the presence of cyclosporin A, which was added to prevent the IMM permeabilization, an essentially monophasic curve of the Ca^{2+} uptake was observed in the presence of otobaphenol (Fig. 8B, trace b). Cyclosporin A

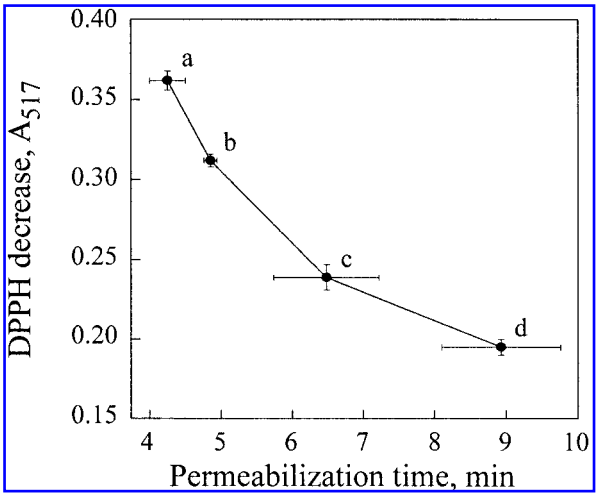

FIG. 7. Relation between the DPPH free radical scavenging activity (decrease of A_{517} for 10 min of incubation) and the permeabilization time of mitochondria (which corresponds to the decrease of A_{540} for 30%) induced by Ca^{2+} in the presence of otobaphenol. Rat liver mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone were added to the MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris. Otobaphenol was added at 0 μ*M* (a), 5 μ*M* (b), 15 μ*M* (c), or 25 μ*M* (d) concentrations to the methanolic media with 30 μ*M* DPPH, or to the 1-min preincubated mitochondria; 30 μ*M* Ca^{2+} was added to the mitochondrial suspension 1 min after otobaphenol.

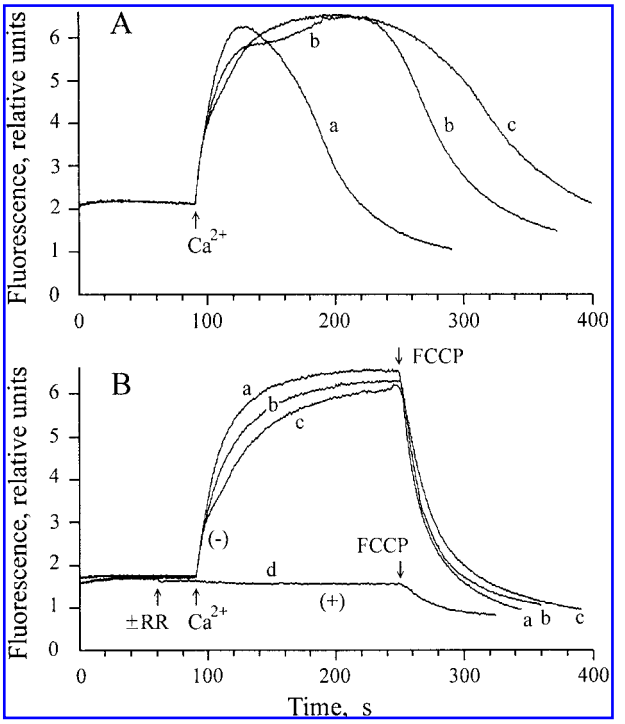

FIG. 8. Influence of otobaphenol and BHT on the chlorotetracycline fluorescence changes related to the input-release of Ca^{2+} into/from rat liver mitochondria. Mitochondria (0.5 mg of protein/ml) and 2.5 μ*M* rotenone with (B) or without (A) 1 μ*M* cyclosporin A were added to the MSH medium supplemented with 10 m*M* acetate-Tris, 5 m*M* succinate-Tris, and 2 μ*M* chlorotetracycline; Ca^{2+}, 100 μ*M* $CaCl_2$; RR, 10 μ*M* ruthenium red (only for trace d); FCCP, 1 μ*M* FCCP; 25 μ*M* otobaphenol (b) or 25 μ*M* BHT (c) was added just after mitochondria. Trace a represents control.

almost did not affect a biphasic character of the chlorotetracycline fluorescence associated with the Ca^{2+} uptake by mitochondria in the presence of BHT (Fig. 8B, trace c). The release of the loaded Ca^{2+}, initiated by FCCP, was somewhat slower in the presence of BHT (Fig. 8B, trace c). The difference in the rate of the Ca^{2+} release was more significant when ruthenium red was added to the Ca^{2+}-loaded mitochondria before FCCP (Fig. 9).

The influence of otobaphenol on the Ca^{2+} uptake by mitochondria was also observed by determining the trans-IMM potential drop as a function of time (Fig. 10). The data show that 25 μ*M* otobaphenol and BHT (Fig. 10, traces b and c, respectively) significantly delayed the Ca^{2+} uptake supported by succinate oxidation in the presence of inorganic phosphate.

The Ca^{2+} uptake in the presence of inorganic phosphate is known to be limited by the respiratory activity of mitochondria. It can be inhibited by uncoupling of mitochondria, by inhibition of the respiratory chain, and by the direct inhibition of the IMM Ca^{2+} uniporter. As shown in Table 2, otobaphenol substantially inhibits the state 3 and the uncoupled state respirations with succinate. No uncoupling effect of both antioxidants was observed, taking into account the unchanged values of the state 4 respiration and the ADP/O ratio (Table 2). The inhibition of the state 3 respiration by otobaphenol also causes a more significant decrease of the respiratory control ratio (RCR) than that by BHT (Table 2).

Thus, the influence of otobaphenol and BHT on the permeability transition pore activation of mitochondria by oxidative stress might result from their effects on the mitochondrial

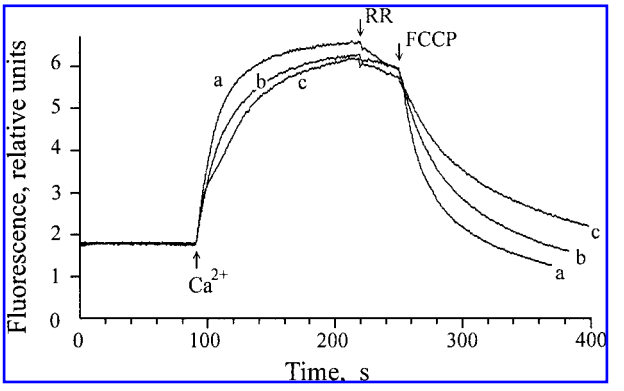

FIG. 9. Influence of otobaphenol and BHT on the ruthenium red-insensitive Ca^{2+} release from rat liver mitochondria. Mitochondria (0.5 mg of protein/ml), 2.5 μ*M* rotenone, and 1 μ*M* cyclosporin A were added to the MSH medium supplemented with 10 m*M* acetate-Tris, 5 m*M* succinate-Tris, and 2 μ*M* chlorotetracycline; Ca^{2+}, 100 μ*M* $CaCl_2$; RR, 10 μ*M* ruthenium red; FCCP, 1 μ*M* FCCP; 25 μ*M* otobaphenol (b) or 25 μ*M* BHT (c) was added just after mitochondria. Trace a represents control.

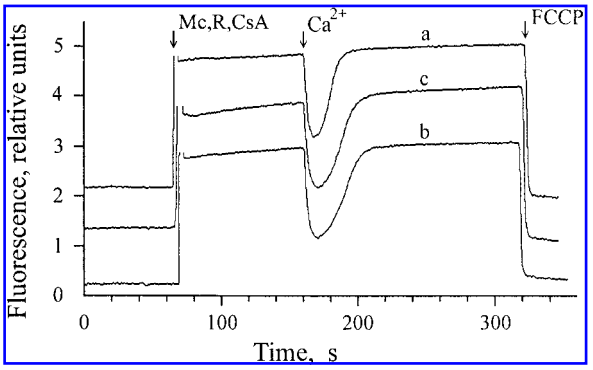

FIG. 10. Influence of otobaphenol and BHT on the time-dependent change of the trans-IMM potential coupled to the Ca^{2+} uptake by rat liver mitochondria. Mitochondria (0.5 mg of protein/ml), 2.5 μ*M* rotenone, and 1 μ*M* cyclosporin A (Mc,R,CsA) were added to the MSH medium supplemented with 5 m*M* phosphate-Tris, 5 m*M* succinate-Tris, and 10 μ*M* safranin O; Ca^{2+}, 100 μ*M* $CaCl_2$; FCCP, 1 μ*M* FCCP; 25 μ*M* otobaphenol (b) or 25 μ*M* BHT (c) was added just after mitochondria. Trace a represents control.

lipoperoxidation, their different free radical scavenging activities, and a partial inhibition of mitochondrial Ca^{2+} transport systems and of the respiratory chain, and even from a direct allosteric action.

DISCUSSION

Oxidative stress can induce the mitochondrial permeability transition causing the swelling of mitochondria and their outer membrane rupture (12, 21, and references therein). Many lines of evidence indicate that antioxidants, as well as metabolic states favoring NAD(P)H and GSH regeneration, which maintains the functioning of the NADPH-GSH-dependent antioxidant enzyme system, substantially delay mitochondrial damages induced by free radicals (1, 4, 12, 21, 31).

Natural phenolic compounds are powerful antioxidants (5). Phenolic resins extracted from various plants of the Myristicaceae family have been shown to possess a comparable or even higher degree of antioxidant activity than BHT (13). In the present work, the antioxidant activity of purified otobaphenol, isolated from the leaves of *Virola Aff. Pavonis* (Myristicaceae) (27), and its influence on the mitochondrial permeability transition pore opening have been studied.

At a concentration of 2.5 μ*M,* otobaphenol completely inhibits the Fe^{3+}-ascorbate-induced lipoperoxidation of the mitochondrial membranes, determined by oxygen consumption (Fig. 1) or by TBA-reactive species accumulation (Table 1). Added to mitochondria with the maximally activated lipoperoxidation, 2.5 μ*M* otobaphenol inhibits it to the same extent as that observed in the presence of 2.5 μ*M* BHT (Fig. 1, traces f and g, respectively). The inhibition by otobaphenol developed significantly more slowly than that by BHT. On the other hand, the DPPH free radical scavenging activity of otobaphenol in methanolic media (Fig. 2A) is significantly higher than that of BHT (Fig. 2B).

The present data also show two different effects of otobaphenol on light scattering by rat liver mitochondria. First, 25 μ*M* and 50 μ*M* otobaphenol substantially delayed the spontaneous swelling of mitochondria incubated in isotonic MSH medium with succinate and inorganic phosphate (Fig. 3, traces b and c). Second, the aggregation of mitochondria was observed at 75 μ*M* or higher concentrations of otobaphenol (Fig. 3, traces d–f). The aggregation of otobaphenol itself was also observed in this medium without mitochondria (Fig. 3, trace g). Thus, trace f in Fig. 3, for example, seems to be simply an approximate superposition of two processes: of the exponential aggregation of mitochondria caused by otobaphenol and of the aggregation of otobaphenol itself (Fig. 3, trace g). The aggregates of otobaphenol itself and the aggregates "mitochondria-otobaphenol" were observed by light microscopy (Fig. 4A and B, respectively). No aggregation of mitochondria occurred when they were added to the medium with the formed aggregates of otobaphenol (Fig. 3, trace g). Moreover, some protective effect of the formed aggregates on mitochondria against oxidative stress was observed (data not shown). The aggregation of mitochondria caused by otobaphenol seems not to be related to its antioxidant activity, because otobaphenol completely inhibits lipoperoxidation at a concentration of 2.5 μ*M,* but even at 25 μ*M,* it does not yet cause an aggregation.

Otobaphenol significantly protected rat liver mitochondria against permeabilization induced by 0.3 m*M* *t*-BOOH or 30

TABLE 2. INFLUENCE OF OTOBAPHENOL AND BHT ON THE RESPIRATION RATE, RCR, AND ADP/O RATIO

Measured parameter	*Control*	*25 μM otobaphenol*	*25 μM BHT*
State 4	62 ± 4	62 ± 2	64 ± 4
State 3	275 ± 16	196 ± 10	240 ± 15
Uncoupled state	360 ± 18	273 ± 10	337 ± 21
RCR	4.46 ± 0.30	3.19 ± 0.11	3.74 ± 0.09
ADP/O	1.57 ± 0.09	1.47 ± 0.02	1.50 ± 0.06

The respiration rate (nmol of O/min/mg of protein), RCR, and ADP/O ratio of rat liver mitochondria were determined (MSH medium supplemented with 5 m*M* succinate-Tris and 5 m*M* phosphate-Tris; 1 mg of mitochondrial protein/ml; 2.5 m*M* rotenone; 0.25 m*M* ADP or 0.1 m*M* DNP added for the state 3 or the uncoupled state respirations, respectively). ($n = 4$)

μ*M* Ca^{2+} (Fig. 5). It also delayed the second phase of endogenous NAD(P)H oxidation induced by 0.3 m*M* *t*-BOOH (Fig. 6A), as well as oxidation of the total pool of NAD(P)H activated by 30 μ*M* Ca^{2+} (Fig. 6B). We have suggested that the second phase of mitochondrial NAD(P)H oxidation induced by *t*-BOOH is realized through the external, rotenone-insensitive pathway of NADH oxidation (24). The second phase occurs when the inner membrane becomes permeable to endogenous pyridine nucleotides (24) and the outer membrane is ruptured due to the mitochondrial swelling (23, 24). The time period between the first and the beginning of the second phase was suggested to be a stage of the permeability transition pore activation by free radicals, because it was essentially delayed by antioxidants (24). This stage is also sensitive to EGTA, but not to desferrioxamine (24), suggesting that Ca^{2+} activates the generation of free radicals, including NO followed by the peroxynitrite production, as has been shown (16, 38, and references therein). The generation of reactive oxygen species in mitochondria in the presence of Ca^{2+} was demonstrated previously (20). In the present work, we observed that BHT was more effective than otobaphenol in delaying the second phase of NAD(P)H oxidation induced by *t*-BOOH (Fig. 6A) and the oxidation of the total pool of NAD(P)H induced by Ca^{2+} (Fig. 6B). On the other hand, BHT causes toxic effects resulting in hepatocellular necrosis, aggregation of chromatin material, and appearance of withered and autolyzed mitochondria (32).

It has been discovered that Ca^{2+} is a powerful activator of mitochondrial NO synthesis (15, 16), whereas *N*-monomethyl-L-arginine, a specific inhibitor of the mitochondrial NO synthase, delays the efflux of Ca^{2+} from the Ca^{2+}-loaded mitochondria (16, 38). As well, the arginine reagent phenylglyoxal essentially inhibits the mitochondrial permeabilization (14). Peroxynitrite was suggested to be a major product of utilization of mitochondrial NO, as a result of recombination of NO and superoxide anion free radicals (35). It has been shown that peroxynitrite induces Ca^{2+} efflux from mitochondria and causes the IMM permeability transition (33). The latter is known to result in mitochondrial swelling and cytochrome *c* release from mitochondria, thus switching on the cell-death program (12, 16, 30, 37). It is not excluded that NO and/or peroxynitrite are the most direct activators of the permeability transition pore, for example, through the tyrosine nitration of the IMM (5, 35). High peroxynitrite scavenging activity of various plant phenols has been demonstrated in the laboratory of Boveris (5), in addition to their NO, hydroxyl radical, and superoxide anion scavenging activities (see 5 for references). Thus, the protective effect of otobaphenol on mitochondria, observed in our work, might be at least partially caused by its free radical scavenging activity.

The opening of the permeability transition pore is caused by synergistic action of Ca^{2+}, inorganic phosphate, and prooxidants (3, 12, 18, 21, 22, 39). The inhibition of the IMM Ca^{2+} uniporter by ruthenium red (10) or external chelating of Ca^{2+} (7) has been shown to prevent the mitochondrial permeability transition. Our data show that both otobaphenol and BHT partially inhibit the mitochondrial Ca^{2+} uptake. This inhibitory action of BHT was higher than that of otobaphenol (Figs. 8 and 9).

The rate of Ca^{2+} uptake may also be limited by the respiratory chain activity, which was more significantly inhibited by otobaphenol than by BHT (Table 2). Both antioxidants decreased the RCR, which was significantly lower in the presence of otobaphenol, but they did not affect the state 4 oxidation rate and the ADP/O ratio (Table 2). As a result of the combined inhibition of the Ca^{2+} uptake and respiration, the time period needed to accumulate 200 nmol of Ca^{2+}/mg of protein of mitochondria was almost equally increased by both antioxidants (Fig. 10).

The observed difference in the effects of otobaphenol and BHT on the mitochondrial permeability transition might partially relate to the inhibition of the cyclic Ca^{2+} transport across the IMM. As was observed, BHT inhibits more effectively than otobaphenol the ruthenium red-insensitive Ca^{2+} release from deenergized mitochondria (Fig. 9). This pathway of the Ca^{2+} release seems to be due to the $Ca^{2+}/2H^{+}$ exchange, because the electrogenic Ca^{2+} uniporter was completely inhibited by ruthenium red. The activation of the $Ca^{2+}/2H^{+}$ exchange by oxidative stress and by Ca^{2+} overload of mitochondria is known to cause the trans-IMM potential drop due to Ca^{2+} cycling across the IMM.

The obtained data show that otobaphenol is a powerful antioxidant and substantially protects mitochondria against permeability transition induced by *t*-BOOH and by Ca^{2+}, although BHT is more effective. Besides antioxidant properties and inhibition of mitochondrial Ca^{2+} transport, an additional protective capacity of BHT might be due to its allosteric effect on the permeability transition pore (17, 36). It has been shown that one nonphenolic BHT derivative, 3,5-di-*tert*-butyltoluene, which is not an antioxidant, also protects rat liver mitochondria against permeability transition (17). It is not known whether this derivative is able to inhibit the $Ca^{2+}/2H^{+}$ exchange through the IMM that was observed for BHT (Fig. 9). On the other hand, the delay of the mitochondrial permeability transition was also observed at concentrations of otobaphenol significantly higher than needed to complete inhibition of the mitochondrial lipoperoxidation induced by Fe^{3+}-ascorbate, like that reported for BHT (17). But a good nonlinear correlation between the mitochondrial protection and the DPPH free radical scavenging activity of otobaphenol, determined in the methanolic medium, was observed for the same range of concentrations of otobaphenol (Fig. 7). Extremely low DPPH free radical scavenging activity of these antioxidants was observed in DMSO, instead of methanol (data not shown). Thus, it is not excluded that the free radical(s) controlling the permeability transition pore is located in the membrane in a microenvironment like methanol.

Although otobaphenol substantially inhibits the mitochondrial respiration, it does not affect the energy coupling of mitochondria and protects them against oxidative stress. Another property of this lignan is that it can cause mitochondrial aggregation at concentrations one order of magnitude higher than that which demonstrated the antioxidant activity or the mitochondrial protection. At these high concentrations, otobaphenol also aggregates itself. The formed aggregates do not cause the aggregation of subsequently added mitochondria, but even somewhat protect them against oxidative stress-induced permeability transition. Thus, otobaphenol and its

derivatives, as well as other plant phenols (5), seem to be promising targets for many practical applications.

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ABBREVIATIONS

BHT, butylated hydroxytoluene; *t*-BOOH, *tert*-butyl hydroperoxide; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; IMM, inner mitochondrial membrane; MSH medium, medium containing 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES-Tris, pH 7.4; NO, nitric oxide; RCR, respiratory control rate; TBA, 2-thiobarbituric acid.

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