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Antioxidant Properties of 2,3-Dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (Idebenone)

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Idebenone [2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone] is a synthetic analogue of coenzyme Q that is currently employed in the treatment of vascular and degenerative diseases of the central nervous system. There is some evidence to suggest that idebenone might function as an antioxidant; however, it has not been demonstrated whether this function pertains to the quinone or hydroquinone form of idebenone. Here we demonstrate that idebenone can scavenge a variety of free radical species, including organic radicals such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and diphenylpicrylhydrazyl, peroxyl and tyrosyl radicals, and peroxynitrite. Idebenone can also redox couple with hypervalent species of Mb or Hb, thus preventing lipid peroxidation promoted by these species. Likewise, idebenone inhibits microsomal lipid peroxidation induced by ADP−iron complexes or organic hydroperoxides. In so doing, idebenone prevents the destruction of cytochrome P450, which otherwise would accompany lipid peroxidation. Irrespective of the experimental system under investigation, idebenone functions by virtue of the electron-donating properties of the hydroquinone form. Redox coupling of this hydroquinone with free radicals generates the quinone compound, which per se lacks antioxidant activity. In many experiments, the antioxidant effects of idebenone become appreciable at ∼2 μM, which is well in the range of plasma levels attainable in patients given oral doses of this drug. Moreover, comparative experiments have shown that the antioxidant efficiency of idebenone varies from no less than 50% to slightly more than 100% of that of vitamin E or Trolox. We would therefore propose that the neuroprotective effects of idebenone can be attributed, at least in part, to its ability to function as an antioxidant, involving redox cycling between hydroquinone and quinone.

Introduction

Several neurodegenerative disorders are thought to originate from the generation of free radicals in excess of antioxidant defenses (*1, 2*). Idebenone [2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone] is a synthetic analogue of coenzyme Q that is currently employed to improve cognitive status in patients with clinical history of stroke, Alzheimer's disease, and multiinfarct dementia (*3, 4*). Despite clinical use, little is known on the mode of action of idebenone. Some investigators have suggested that idebenone improves cerebral energy metabolism (*4, 5*) and stimulates nerve growth factor synthesis (*4, 6*). Others (*7−11*) have ascribed the neuroprotective action of idebenone to antioxidant properties; in fact, idebenone inhibits lipid peroxidation in brain homogenates (*7*), mitochondrial membranes (*8*), and neural cell lines subjected to oxidative stress (*9*). Idebenone also appears to minimize platelet formation of thromboxane (*10*) as well as the toxicity of oxidized low-density lipoprotein to endothelial cells (*11*). In so doing, idebenone inhibits platelet aggregation (*10*) and contributes to the maintenance of vascular wall integrity and functions (*11*).

Idebenone may exist as quinone (IDB[1]) and hydroquinone ($IDBH_2$) forms (Chart 1); however, it has not been established which form is endowed with antioxidant activity (*7−12*). The present work was therefore intended to characterize the antioxidant properties of $IDBH_2$ versus IDB in a variety of in vitro free radical systems. We demonstrate that idebenone is a rather effective antioxidant which stands comparison with coenzyme Q, vitamin E, or its water-soluble analogue Trolox. Irrespective of the experimental system under investigation, idebenone functions by virtue of the electron-donating properties of $IDBH_2$; IDB per se lacks antioxidant activity and is formed as a byproduct of the redox coupling between $IDBH_2$ and free radicals. We would

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[1] Abbreviations: $IDBH_2$, idebenone hydroquinone; IDB, idebenone quinone; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; (*R*)-PE, (*R*)-phycoerythrin; PnA, 9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid, *cis*-parinaric acid; DHR, dihydrorhodamine-123; H_2O_2, hydrogen peroxide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); $ABTS^{\bullet+}$, ABTS cation radical; PC, phosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; $O_2^{\bullet-}$, superoxide anion; Mb^{IV}, ferrylmyoglobin; $^{\bullet}Mb^{IV}$, ferrylmyoglobin radical; Mb^{III}, metmyoglobin; $Mb^{II}O_2$, oxymyoglobin; TBARS, thiobarbituric acid-reactive substances; $^{\bullet}NO$, nitric oxide; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; $CoQ_{10}H_2$, coenzyme Q_{10} hydroquinone; CUOOH, cumene hydroperoxide; LMW, low molecular weight.

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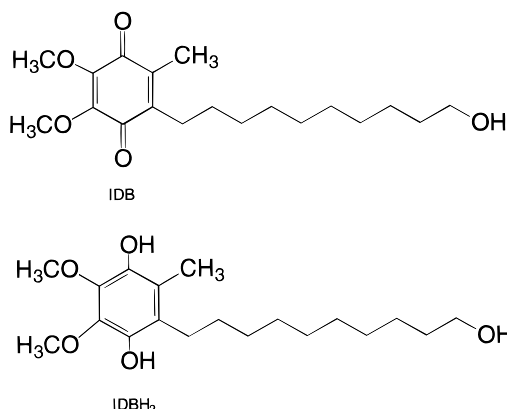
Chart 1. Structures of Quinone (IDB) and Hydroquinone ($IDBH_2$) Idebenone



therefore propose that the neuroprotective effects of idebenone can be attributed, at least in part, to its ability to function as an antioxidant, involving redox cycling between $IDBH_2$ and IDB.

Materials and Methods

Chemicals. Idebenone quinone (IDB) was kindly provided by Takeda Pharmaceutical Co. (Tokyo, Japan). $IDBH_2$ (ϵ_{290nm}= 3.94 mM^{-1} cm^{-1}) was prepared by chemical reduction of IDB (ϵ_{275nm} = 14 mM^{-1} cm^{-1} in absolute ethanol), essentially as described by Rieske for short-chain coenzyme Q analogues (*13*). Purity was checked by reverse-phase HPLC. Stock solutions of $IDBH_2$ were stored in the dark at −20 °C, in absolute ethanol under acidic conditions (10 mM HCl).

(*R*)-Phycoerythrin [(*R*)-PE] and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Polysciences Inc. (Warrington, PA).

Caution: *AAPH is toxic and is an irritant. Use care in handling*.

cis-Parinaric acid (PnA) and dihydrorhodamine-123 (DHR) were obtained from Molecular Probes (Eugene, OR). Hydrogen peroxide (H_2O_2), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Trolox were from Aldrich Chemical Co. (Milwaukee, WI). Beef liver catalase (E.C. 1.11.1.6) was from Boehringer Mannheim (Germany) and was made free of thymol by ultrafiltration in Diaflo YM100 membrane (Amicon, Beverly, MA). Saffron, egg yolk phosphatidylcholine (PC), cumene hydroperoxide (CUOOH), arachidonic acid, horse heart myoglobin (type III, 99% purity), and bovine hemoglobin were from Sigma Chemical Co. (St. Louis, MO). Crocin was isolated with methanol from saffron, after treatment with ethyl ether to remove interfering substances (*14*). The concentration of crocin was determined by assuming ϵ_{443nm} = 133 mM^{-1} cm^{-1} (*14*). All solutions were prepared in 18 MΩ double-distilled deionized water (Milli-Q, Millipore Co., Bedford, MA). Unless otherwise indicated, the reactions were carried out in a standard buffer composed of 50 mM sodium phosphate buffer, pH 7.4. Phosphate buffer was routinely passed through Chelex-100 (Bio-Rad) to minimize metal contamination. DTPA (0.1 mM) was also included in the standard buffer to prevent possible interferences from residual trace metals.

Assays. 1. DPPH and ABTS Radical Scavenging. The stable nitrogen-centered free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was prepared by mixing 1 mL of standard buffer with 1 mL of absolute ethanol and 0.5 mL of ethanol-dissolved 500 μM DPPH, at 25 °C (*15*). Following stabilization of absorbance at 516 nm, antioxidants were included and the concentrations inducing a decrease of 0.20 absorbance unit/10 min ($IC_{0.20}$) were assumed as biochemical indices of free radical-scavenging efficiency. The monocation radical of ABTS ($ABTS^{•+}$) was generated by reacting this probe with Mb^{IV} (*16*). Briefly, ABTS (150 μM) was reconstituted with Mb^{III} (2.5 μM) in standard buffer, at 20 °C, in the presence or absence of antioxidants. Hydrogen peroxide (75 μM) was subsequently included to convert Mb^{III} to Mb^{IV}, and the formation of $ABTS^{•+}$ was monitored spectrophotometrically as the absorbance increase at 734 nm. The lag time from H_2O_2 addition to absorbance increase was used to calculate the antioxidant efficiency of test compounds. In a separate set of experiments $ABTS^{•+}$ was generated by electrolysis as described by Barr and Aust (*17*), using a PS500XT power supply (Hoefer Scientific Instruments, San Francisco, CA). $ABTS^{•+}$ was stable at neutral pH, and its concentration after electrolysis was determined by assuming ϵ_{660nm} = 12 mM^{-1} cm^{-1} (*17*).

2. Peroxyl Radical Scavenging. Peroxyl radicals were generated in aqueous environments by thermal decomposition of AAPH (*18*). The peroxyl radical-scavenging activity of idebenone was evaluated from its competition with hydrophilic or hydrophobic probes such as (*R*)-PE (*19*) and crocin (*14*) or PnA (*20*), respectively. In a first system, 1.2 nM (*R*)-PE was equilibrated for 5 min at 37 °C and subsequently reacted with 4 mM AAPH. Peroxyl radical reactivity was then determined from the loss of (*R*)-PE fluorescence (emission at 575 nm, excitation at 495 nm). Where indicated, test compounds (or equivalent volumes of ethanol-HCl) were included 20 min after AAPH addition, and antioxidant efficiencies were determined from their ability to prevent fluorescence decay. In a second system, (*R*)-PE was replaced with crocin, which is bleached by AAPH-derived radicals. Briefly, the reaction mixture contained crocin (12 μM) and increasing amounts of antioxidants or equivalent volumes of ethanol-HCl in standard buffer, 40 °C. The reaction was started by the addition of 5 mM AAPH, and the bleaching of crocin was monitored as the decrease in absorbance at 440 nm (ϵ = 133 mM^{-1} cm^{-1}) (*14*). A linear bleaching rate was detected approximately 30 s after the addition of AAPH and lasted several minutes. Experimental data were eventually plotted to fit the following competition kinetic equation:

$$V_c/V_a = 1 + K_a/K_c[A]/[C]$$

where V_c is the crocin bleaching rate in the absence of antioxidant, V_a is the crocin bleaching rate in the presence of antioxidant, K_a is the rate constant for the interaction of free radicals with the antioxidant, K_c is the rate constant for the reaction between free radicals and crocin, [A] is the antioxidant concentration, and [C] is the crocin concentration. The K_a/K_c slope, calculated from the linear regression of the plot of [A]/[C] vs V_c/V_a, indicates the relative capacity of antioxidants to interact with peroxyl radicals.

In a third system, idebenone was incorporated in PC liposomes which also contained PnA, a fluorescent polyunsaturated fatty acid which is suitable to monitor the early phases of lipid peroxidation in liposomal vesicles (*20*), ghosts, and intact erythrocytes (*21*). The preparation of PC liposomes and the incorporation of PnA, idebenone, or other antioxidants were as described previously (*22*). Typical incubations (3 mL final volume) consisted of 200 μM PC liposomes containing 1.5 μM PnA (corresponding to a 0.75% molar ratio to phospholipids) and increasing concentrations of antioxidants in standard buffer, 37 °C. Lipid peroxidation was initiated with 10 mM AAPH, and the oxidative modifications of PnA were monitored as fluorescence decay (excitation at 324 nm, emission at 413 nm).

3. Studies with Tyrosyl Radicals. UV irradiation of tyrosine solutions generates tyrosyl radicals which subsequently recombine to fluorescent dityrosines (*23*). L-Tyrosine (400 μM) was incubated in standard buffer at 25 °C in the presence or absence of idebenone. Superoxide dismutase (70 units mL^{-1}) and catalase (500 units mL^{-1}) were included to remove $O_2^{•-}$ formed during irradiation and H_2O_2 formed via $O_2^{•-}$ dismuta-

tion, respectively. UV irradiation was carried out at 275 nm, and dityrosine fluorescence emission was recorded at 405 nm. The scavenging of tyrosyl radicals by test compounds was inferred from the delay in the fluorescence buildup.

4. Interactions with Hypervalent Hemeproteins: Spectral Studies and Lipid Peroxidation Experiments. The formation of Mb^{IV} or Hb^{IV} was monitored in incubations (2 mL final volume) containing Mb^{III} or Hb^{III} (both adjusted to give 50 μM heme) and H_2O_2 (150 μM) (*24*). Mb^{IV} concentration was determined by measuring absorbance at 550 and 630 nm, according to the following formula: $[Mb^{IV}]$ (μM) = [249 $\times$ absorbance at 550 nm] − [367 $\times$ absorbance at 630 nm] (*25*). After idebenone addition the Mb^{IV}-to-Mb^{III} transition was monitored as the absorbance decrease at 550 nm, that is the wavelength at which the absorption spectra of Mb^{III} and Mb^{IV} differ most (*24*). Concurrent oxidation of $IDBH_2$ was monitored at 275 nm by assuming ϵ = 12.5 mM^{-1} cm^{-1} for [IDB] *minus* [$IDBH_2$]. At this wavelength the spectral difference between Mb^{III} and Mb^{IV} is minimal and does not interfere with IDB determination. In all experiments the addition of idebenone was routinely preceded by that of catalase (500 units mL^{-1}). This was intended to decompose unreacted H_2O_2 which otherwise would convert Mb^{III} back to Mb^{IV}, altering the apparent stoichiometry of Mb^{IV} reduction and idebenone oxidation.

Ferrylmyoglobin- or ferrylhemoglobin-dependent lipid peroxidation was studied in incubations containing arachidonic acid vesicles (0.4 mM) and Mb^{III} or Hb^{III} (50 μM) in standard buffer at 37 °C. Reactions were started by adding H_2O_2 (150 μM), and lipid peroxidation was assayed as the formation of thiobarbituric acid-reactive substances (TBARS) (*26*), with modifications involving butanol extraction of the TBA adduct (*27*). In other experiments, lipid peroxidation was monitored as the fluorescence decay of PC liposome-incorporated PnA. Briefly, a small volume of an ethanolic solution of PnA (final concentration 1 μM, corresponding to a 0.5% molar ratio to phospholipids) was injected into reaction mixtures (3 mL final volume) containing 200 μM phospholipid in standard buffer at 20 °C. Lipid peroxidation was initiated by adding Mb^{III} (2 μM) and H_2O_2 (5 μM). Where indicated, the addition of Mb^{III} and H_2O_2 was preceded by that of antioxidants. The loss of PnA fluorescence was monitored with excitation at 312 nm and emission at 455 nm.

5. Studies with Peroxynitrite. Peroxynitrite ($ONOO^-$) is formed by reaction of superoxide anion ($O_2^{\bullet-}$) with nitric oxide ($^{\bullet}NO$). In this study $ONOO^-$ was generated by the spontaneous decomposition of 3-morpholinosydnonimine (SIN-1) at neutral pH, yielding $O_2^{\bullet-}$ and $^{\bullet}NO$. $ONOO^-$ formation was monitored spectrophotometrically by taking advantage of its ability to oxidize DHR to rhodamine, having a high molar extinction coefficient at 500 nm (74 500 M^{-1} cm^{-1}) (*28*). Neither $O_2^{\bullet-}$ nor H_2O_2 or $^{\bullet}NO$ per se can oxidize DHR, thus making this assay specific for $ONOO^-$ (*28*). The reagent mixture (2 mL final volume) consisted of DHR (50 μM) and SIN-1 (1 mM) in standard buffer, 30 °C. Antioxidants or equivalent volumes of ethanol-HCl were added 15 min after SIN-1, i.e., when the rate of $ONOO^-$ formation was constant.

6. ADP−Iron-Dependent Microsomal Lipid Peroxidation and Cytochrome P450 Destruction. Microsomes were isolated from the livers of male Wistar rats (~200 g body wt) which had been given phenobarbital intragastrically for 3 days at a daily dose of 20 mg/kg body wt. Phenobarbital treatment had the dual advantage of inducing cytochrome P450 (a major catalyst of microsomal lipid peroxidation) and depleting a membrane pool of non-heme iron which participates in free radical reactions and potentially interferes with exogenously added iron or other pro-oxidant agents (*29*, *30*). Microsomes were isolated as described previously (*31*) and assayed for proteins and cytochrome P450 by established procedures (*32*, *33*). Lipid peroxidation was studied by reconstituting microsomes (1 mg of protein/mL) with ADP−Fe(II) (1 mM chelator: 0.1 mM $FeSO_4$) or CUOOH (0.25 mM). These experiments were performed in 0.3 M NaCl, carefully adjusted to pH 7.0 just prior to use. This was done to avoid ligand-catalyzed interaction between iron and either phosphate or DTPA present in the standard buffer; similar ligand interactions may occur with several other buffers (*29*). Aliquots of reaction mixtures were taken at the indicated times and assayed separately for TBARS formation or cytochrome P450 destruction. Although unbuffered, the pH of reaction mixtures did not vary throughout the experiment time.

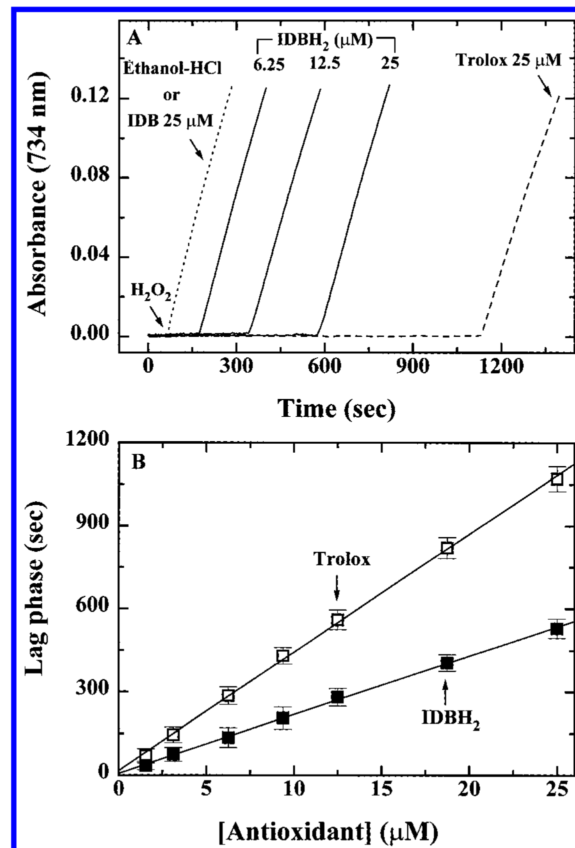


Figure 1. Interaction of idebenone with the ABTS radical. Incubations were prepared as described under Materials and Methods. Panel A shows the time-dependent formation of the ABTS radical in the absence (···) or presence of $IDBH_2$ (—) or Trolox (− −). In panel B the lag phases preceding radical detection were plotted as a function of $IDBH_2$ (■) or Trolox (□) concentration.

Unless otherwise indicated all values are given as means ± SE. Other experimental conditions are given in the legends to figures and tables.

Results

Interaction of Idebenone with DPPH and ABTS Radicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable nitrogen-centered free radical which may convert into 1,1-diphenyl-2-picrylhydrazine, a diamagnetic molecule, upon one-electron reduction. DPPH has a strong absorption at 516 nm which decreases after the addition of reducing equivalents (*15*). Experiments with 2.5−50 μM $IDBH_2$ showed an instantaneous and dose-dependent decrease of DPPH absorbance, whereas similar concentrations of IDB were virtually ineffective. In this system, the efficiency of $IDBH_2$ was very similar to that of Trolox ($IC_{0.200}$ = 12.02 ± 0.54 vs 11.98 ± 0.71 μM; n = 7). Furthermore, the efficiency of $IDBH_2$ was comparable to that of other antioxidants, such as α-tocopherol or butylated hydroxytoluene ($IC_{0.200}$ = 12.77 ± 0.81 and 11.35 ± 0.5 μM, respectively) (*34*).

As shown in Figure 1A (dotted line), the inclusion of H_2O_2 in a mixture containing Mb^{III} and ABTS produced

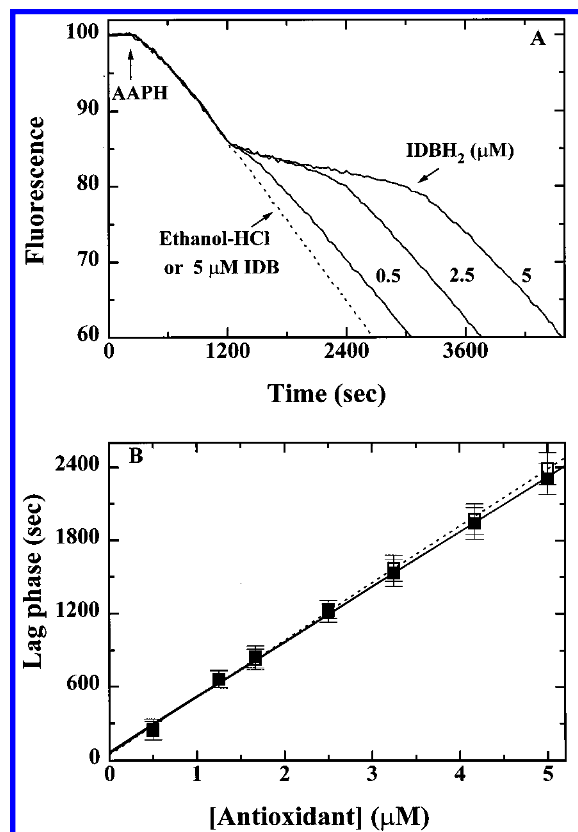


Figure 2. Effect of idebenone on peroxyl radical-dependent oxidations: studies with (*R*)-PE. Peroxyl radicals were generated in incubations containing AAPH, as described under Materials and Methods. Panel A shows the effect of IDB or ethanol-HCl (···) versus increasing concentrations of $IDBH_2$ (—) on the fluorescence decay of (*R*)-PE (1.2 nM) induced by AAPH (4 mM). In panel B the lag phases preceding the fluorescence decay were plotted as a function of $IDBH_2$ (■) or Trolox (□) concentration.

an instantaneous and linear increase of absorbance at 734 nm, indicative of the formation of $ABTS^{•+}$. In the presence of $IDBH_2$ (solid line), the absorbance increase was delayed by a lag phase that increased linearly with the concentration of this drug (Figure 1). By calculating the slope values of straight lines obtained by plotting lag phases versus antioxidant concentrations, we could determine that $IDBH_2$ was 50% as efficient as Trolox in trapping $ABTS^{•+}$ (Figure 1B). In selected experiments, $ABTS^{•+}$ was generated electrochemically and the decay in absorbance at 660 nm was monitored in the presence or absence of $IDBH_2$ or Trolox. Under these conditions, $IDBH_2$ quenched $ABTS^{•+}$ with a 50% efficiency as compared with Trolox, thus confirming the experiments in which this species was generated by ferrylmyoglobin. Neither IDB nor ethanol-HCl was found to scavenge $ABTS^{•+}$ (see also Figure 1A).

Interaction of Idebenone with Peroxyl Radicals. AAPH is a water-soluble azo compound which produces a constant flow of peroxyl radicals upon thermal decomposition. As shown in Figure 2A, incubation of (*R*)-PE with AAPH resulted in a linear decrease of its fluorescence emission (dotted line). When $IDBH_2$ was included 20 min after AAPH addition, the decay in fluorescence stopped and then resumed after a lag phase which increased with the concentration of $IDBH_2$ (see Figure 2A, solid lines, and B, closed symbols, solid line). Considering the rate constant of peroxyl radical generation (1.1×10^{-6} [AAPH] s^{-1} at 37 °C) (*18*) and the length of the lag phase during which (*R*)-PE was protected from oxidation, we determined that one $IDBH_2$ could trap two peroxyl radicals (1.94 ± 0.07, $n = 7$). Under the same experimental conditions, a similar stoichiometry (2.06 ± 0.08, $n = 7$) was obtained with Trolox (Figure 2B, open symbols, dotted line). In this assay neither IDB nor an equivalent volume of ethanol-HCl could affect the AAPH-dependent decay of (*R*)-PE fluorescence (see also Figure 2A). Lipophilic antioxidants, such as $CoQ_{10}H_2$ and α-tocopherol, were similarly ineffective, as already reported (*35*).

The ability of $IDBH_2$ to scavenge AAPH-derived peroxyl radicals was further assessed by the crocin bleaching test. This assay has the advantage of providing some indirect information on the nature of byproducts eventually formed through reaction of antioxidants with peroxyl radicals. In fact, the bleaching rate can paradoxically increase if antioxidant generates byproducts that are themselves capable to react with crocin, as demonstrated with Ebselen (*36*). By contrast, the bleaching rate will decrease if antioxidant byproducts remain unreactive or contribute to intercept peroxyl radicals, as demonstrated with Trolox (*36*). In preliminary experiments we found that 6.25 or 12.5 µM $IDBH_2$ decreased the rate of peroxyl radical-dependent crocin bleaching from 0.316 to 0.236 or 0.183 nmol/(mL·min)$^{-1}$, respectively. The reaction was then investigated within a broad range of $IDBH_2$ concentrations (2.5−25 µM), and the bleaching rates were plotted according to the competition kinetic equation described under Materials and Methods. A K_a/K_c ratio was calculated and used as an index of the efficiency with which antioxidants reacted with peroxyl radicals. Under these conditions, the K_a/K_c for $IDBH_2$ was virtually identical with that calculated for Trolox (0.753 ± 0.045 vs 0.788 ± 0.055, respectively; $n = 6$), suggesting a comparable efficiency in peroxyl radical scavenging.

The above-described experiments focused on the ability of $IDBH_2$ to protect hydrophilic probes such as (*R*)-PE and crocin from peroxyl radicals generated by thermal decomposition of AAPH. In a subsequent set of experiments both the oxidizable probes and $IDBH_2$ were incorporated in a lipid environment. For this purpose, we used PnA, a polyunsaturated fatty acid which becomes fluorescent upon incorporation in lipid bilayers. The decay of PnA fluorescence is therefore indicative of peroxidative processes within lipid environments. As shown in Figure 3A, the fluorescence of PnA incorporated in PC liposomes decreased linearly upon exposure to AAPH. Figure 3A also shows that simultaneous incorporation of an increasing amount of $IDBH_2$ in PC liposomes prevented the decay of PnA fluorescence, suggesting that $IDBH_2$ protected PnA from peroxyl radicals. To permit adequate evaluation of the antioxidant activity of $IDBH_2$ in lipid environments, comparative experiments were performed with PC liposomes fortified with vitamin E. These experiments showed that $IDBH_2$ was slightly but significantly more effective than α-tocopherol in protecting PnA from AAPH-dependent oxidation (Figure 3B).

Interaction of Idebenone with Tyrosyl Radical. The tyrosyl radical-scavenging activity of idebenone was studied in a model system relying on UV irradiation of aqueous tyrosine solutions, yielding tyrosyl radicals which rearrange to fluorescent dityrosines (*23*). In this system, compounds that donate electrons to tyrosyl radicals suppress or delay the formation of fluorescent

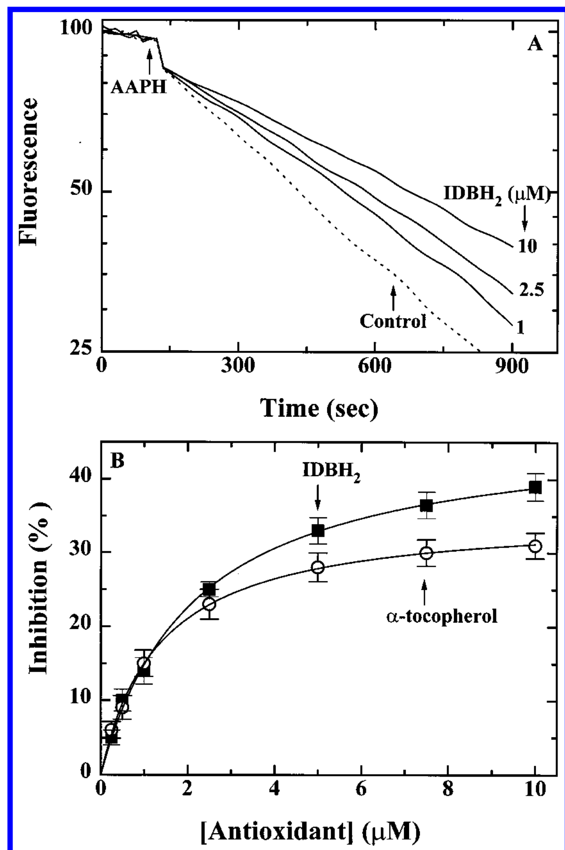


Figure 3. Effect of idebenone on peroxyl radical-dependent oxidations: studies with PnA. Incubations were prepared as described under Materials and Methods. Panel A shows the AAPH-dependent decrease in fluorescence of liposome-incorporated Pna in the absence (···) or presence of 1, 2.5, or 10 μM $IDBH_2$ (—). In panel B the inhibition of PnA fluorescence decay was plotted as a function of $IDBH_2$ (■) or α-tocopherol (○) concentration.

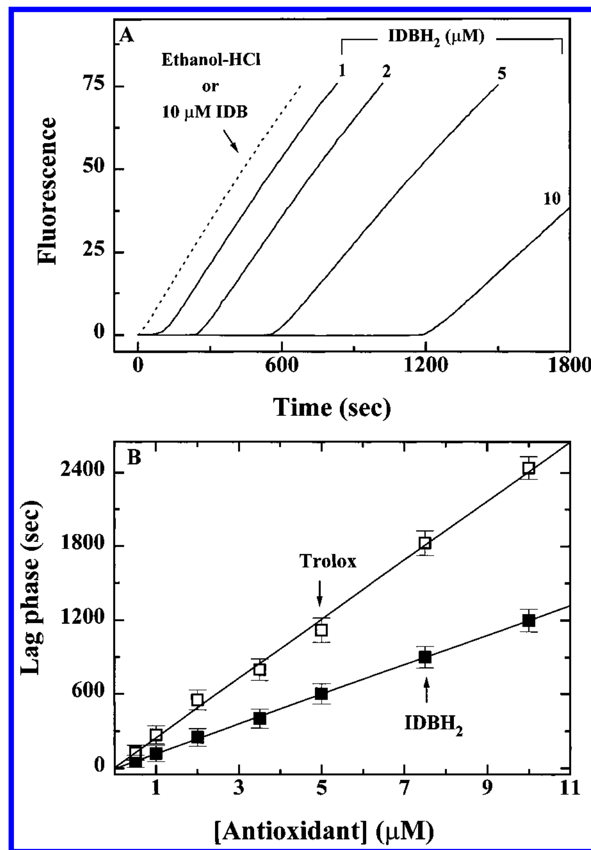


Figure 4. Effect of idebenone on dityrosine fluorescence buildup. Incubations were prepared as described under Materials and Methods. Panel A shows the dityrosine fluorescence buildup in the absence (···) or presence of 1, 2.5, 5, or 10 μM $IDBH_2$ (—). In panel B the lag phases preceding the buildup of fluorescence were plotted as a function of $IDBH_2$ (■) or Trolox (□) concentration.

dityrosines. As shown in Figure 4A (dotted line), UV irradiation of tyrosine produced an immediate and linear increase of fluorescence emission. In the presence of $IDBH_2$ (solid line) the fluorescence buildup was delayed by the appearance of a lag phase that increased with the concentration of the antioxidant (Figure 4). The slope values of the corresponding straight lines showed that $IDBH_2$ was 50% as efficient as Trolox in scavenging tyrosyl radical. Neither IDB nor ethanol-HCl was found to inhibit dityrosine formation (see also Figure 4*A*).

Interaction of Idebenone with Ferrylhemoproteins. In the presence of H_2O_2, Mb^{III} and Hb^{III} undergo a two-electron oxidation process yielding a transient protein radical *plus* a long-lived oxoferryl moiety (Fe^{IV}=O), which can be detected spectrophotometrically as changes in the visible and Soret regions. As shown in Figure 5A, the spectrum of Mb^{III} (dashed line) was characterized by absorption peaks at 502, 582, and 632 nm. The addition of a 3-fold excess of H_2O_2 (Figure 5*A*, dotted line) induced a rapid absorbance increase in the 520–600-nm region with distinct peaks at 546 and 586 nm, *plus* disappearance of the peak at 632 nm. These spectral changes have been attributed to the formation of Mb^{IV} or an $^{\bullet}Mb^{IV}/Mb^{IV}$ composite (*25, 37*). After catalase removal of unreacted H_2O_2, the addition of $IDBH_2$ caused spectral changes consisting of disappearance of the peaks at 546 and 586 nm *plus* reappearance of the peaks at 502 and 582 nm (Figure 5A, solid line). This spectral pattern was therefore similar to that of native Mb^{III} (cf. Figure 5A, dashed line) with the exception that the 632-nm peak was shifted to 622 nm. This spectral shift has already been observed upon reduction of Mb^{IV} by other electron donors (*24, 25, 37*). Under the same experimental conditions, neither IDB nor ethanol-HCl was able to reduce Mb^{IV} (not shown).

Spectra recorded in the Soret region confirmed the data obtained in the visible region. The spectrum of Mb^{III} in the Soret region was characterized by an absorption peak at 408 nm (Figure 5A inset, dashed line). The addition of a 3-fold excess of H_2O_2 induced a shift from 408 to 420 nm (Figure 5A inset, dotted line), which is distinctive of Mb^{IV} (*37*). After catalase removal of unreacted H_2O_2, reconstitution of $IDBH_2$ with Mb^{IV} shifted the peak at 420 nm back to 408 nm (see also Figure 5A inset, solid line), confirming the reductive action of $IDBH_2$ on Mb^{IV}.

The Mb^{IV}-to-Mb^{III} transition was also monitored as the decrease in absorbance at 550 nm. As shown in Figure 6*A* neither IDB nor an equivalent volume of ethanol-HCl could reduce Mb^{IV}. By contrast, $IDBH_2$ irreversibly and concentration-dependently reduced Mb^{IV} to Mb^{III} (Figure 6). Moreover, the initial rate of Mb^{III} formation was directly proportional to the concentration of $IDBH_2$, with a linear relationship in the 1–10 μM range (Figure 6B inset). The apparent second-order rate constant for the reaction between $IDBH_2$ and Mb^{IV}, as determined from the linear portion of the plot $d[Mb^{III}]/dt$ vs $[IDBH_2]$, was $3.78 \pm 0.09 \times 10^{-4}\ M^{-1}\ s^{-1}$ ($n = 6$). This value is close to that reported for short-chain CoQ analogues such as CoQ_1H_2 and CoQ_2H_2 (*24*). The reduction of Mb^{IV} to Mb^{III}

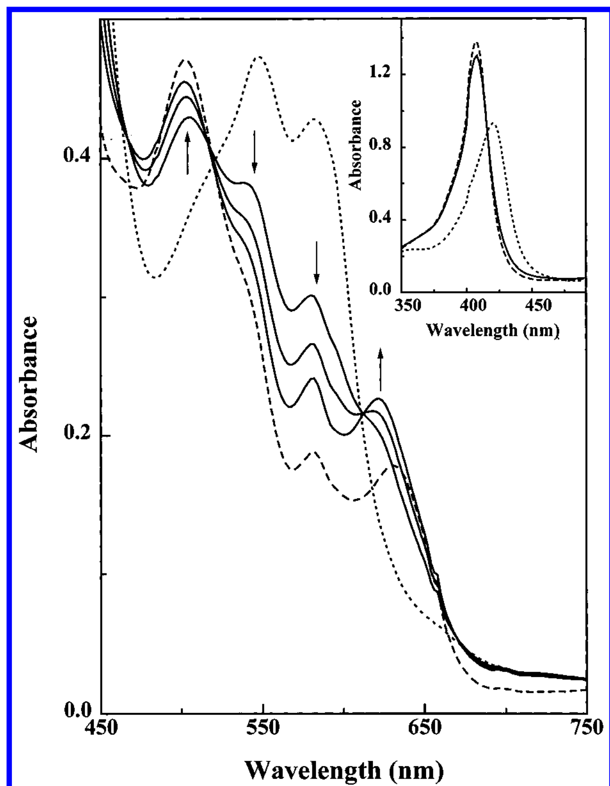


Figure 5. Reduction of Mb^{IV} by idebenone. Ferrylmyoglobin (···) was produced by reacting 50 μM Mb^{III} (– –) with 150 μM H_2O_2. Following the addition of catalase (500 units mL^{-1}) to remove unreacted H_2O_2, 50 μM $IDBH_2$ was included and repetitive scans (—) were taken every 1 min. The inset shows the spectral changes in the Soret region under comparable conditions, with the exception that Mb^{III}, H_2O_2, and $IDBH_2$ concentrations were lowered to 10, 30, and 10 μM, respectively.

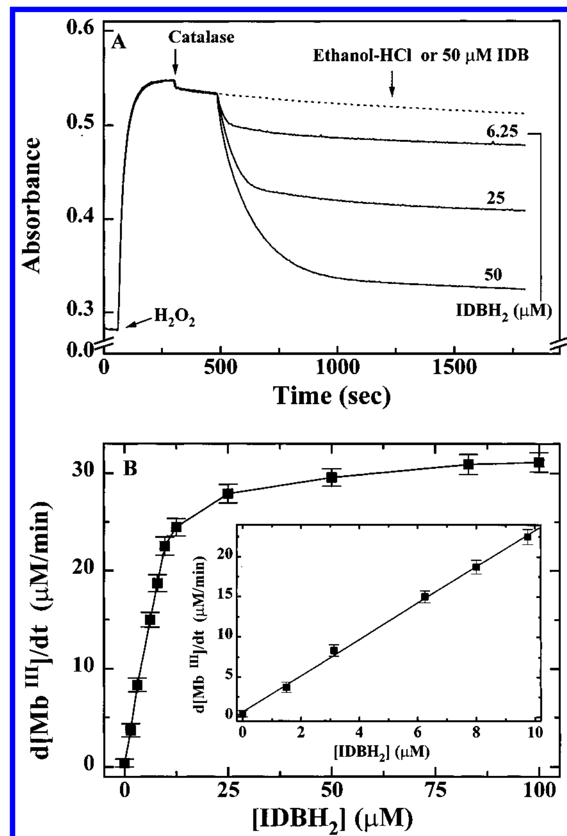


Figure 6. Idebenone-dependent reduction of Mb^{IV}. Ferrylmyoglobin was formed by reacting Mb^{III} with H_2O_2 as described in Figure 5. The reduction of Mb^{IV} to Mb^{III} by $IDBH_2$ versus IDB or ethanol-HCl was monitored as the decrease in absorbance at 550 nm. Panel A shows a time course experiment. Panel B summarizes the dependence of Mb^{III} formation versus idebenone concentration.

was paralleled by the oxidation of $IDBH_2$ to IDB, and the stoichiometry of idebenone oxidation versus Mb^{IV} reduction (as determined from d[Mb^{III}]/dt vs d[oxidized idebenone]/dt) averaged unity (1.03 ± 0.04, n = 6). Comparative experiments showed that $IDBH_2$ could also reduce Hb^{IV}. In fact, 5 min reaction of 100 μM $IDBH_2$ with 50 μM Hb gave a mixture composed of Hb^{IV} (4 μM), Hb^{III} (38 μM), and $Hb^{II}O_2$ (8 μM).

The hypervalent states of hemoproteins are strong oxidants that promote oxidation, peroxidation, and epoxidation of various biomolecules in vitro (*37*). In particular, the oxoferryl moiety of Mb^{IV}, but not the protein radical, has been found to promote the peroxidation of fatty acids, membranes, and lipoproteins (*37*). Antioxidants that donate electron to the oxoferryl moiety will therefore protect against oxidative damage. This has been demonstrated with Trolox, ascorbate, ergothioneine, urate, thiols, and CoQ_1H_2 or CoQ_2H_2 (*24, 25, 37*). As shown in Table 1, the incubation of arachidonic acid with a mixture of Mb^{III} *plus* H_2O_2 yielded TBARS indicative of peroxidation. The addition of $IDBH_2$ decreased lipid peroxidation close to the values observed with either Mb^{III} or H_2O_2 added individually, in keeping with the ability of $IDBH_2$ to compete with lipids for the oxidizing equivalents of H_2O_2-activated Mb. Fully comparable results were obtained in experiments containing Hb^{III} in place of Mb^{III} (see also Table 1).

Further evidence for a redox coupling of $IDBH_2$ with H_2O_2-activated hemoproteins was obtained in a reconstituted system involving PnA incorporated in PC liposomes. As shown in Figure 7A, the addition of Mb^{III} or

Table 1. Effects of Idebenone on Mb^{IV}- or Hb^{IV}-Dependent Lipid Peroxidation

addition	TBARS (nmol/mL)[a]
Mb^{III} + arachidonic acid[b]	0.34 ± 0.01
H_2O_2 + arachidonic acid[b]	0.19 ± 0.01
Mb^{III} + H_2O_2 + arachidonic acid[c]	2.96 ± 0.17
Mb^{III} + H_2O_2 + $IDBH_2$ (100 μM) + arachidonic acid[c]	0.44 ± 0.01
Mb^{III} + H_2O_2 + IDB (100 μM) + arachidonic acid[c]	2.92 ± 0.25
Hb^{III} + arachidonic acid[b]	0.43 ± 0.01
Hb^{III} + H_2O_2 + arachidonic acid[c]	2.92 ± 0.24
Hb^{III} + H_2O_2 + $IDBH_2$ (100 μM) + arachidonic acid[c]	0.47 ± 0.01
Hb^{III} + H_2O_2 + IDB (100 μM) + arachidonic acid[c]	2.86 ± 0.25

[a] Lipid peroxidation products were determined as TBARS versus reagent blanks lacking Mb^{III} or Hb^{III}. Values are means ± SE of three separate determinations in duplicate. [b] Arachidonic acid (0.4 mM) was incubated for 30 min at 37 °C with either horse heart Mb^{III} (50 μM), bovine Hb^{III} (50 μM), or H_2O_2 (150 μM). [c] Horse heart Mb^{III} (50 μM) or bovine Hb^{III} (50 μM) was incubated with H_2O_2 (150 μM) for 5 min at 20 °C. After removal of excess of H_2O_2 by catalase (500 units mL^{-1}), $IDBH_2$ or IDB was included. Arachidonic acid (0.4 mM) was eventually added, and reaction mixtures were incubated for 30 min at 37 °C.

H_2O_2 apparently accompanied with a slow decay of PnA fluorescence; however, control experiments showed that such decay reflected a spontaneous photobleaching of PnA, inasmuch as it could be observed even in the absence of Mb^{III} or H_2O_2. When both Mb^{III} and H_2O_2 were included in the reaction mixtures, a rapid and extensive fluorescence decay was observed. The addition of $IDBH_2$ concentration-dependently prevented the decay, consistent with the ability of this antioxidant to reduce the

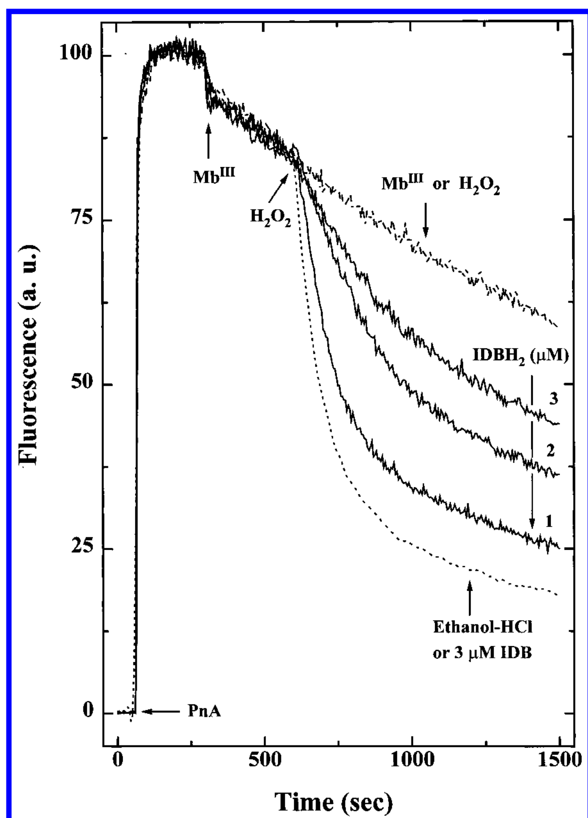


Figure 7. Effect of idebenone on Mb^{IV}-dependent oxidation of PnA incorporated in liposomes. Ferrylmyoglobin was formed by reacting Mb^{III} (2 μM) with H_2O_2 (5 μM). The fluorescence decay of liposome-incorporated PnA was monitored in the presence of 1−3 μM IDBH$_2$ (—) and either 3 μM IDB or an equivalent volume of ethanol-HCl (···), as described under Materials and Methods. The upper trace shows that Mb^{III} or H_2O_2 per se had no effect on the decay of PnA fluorescence.

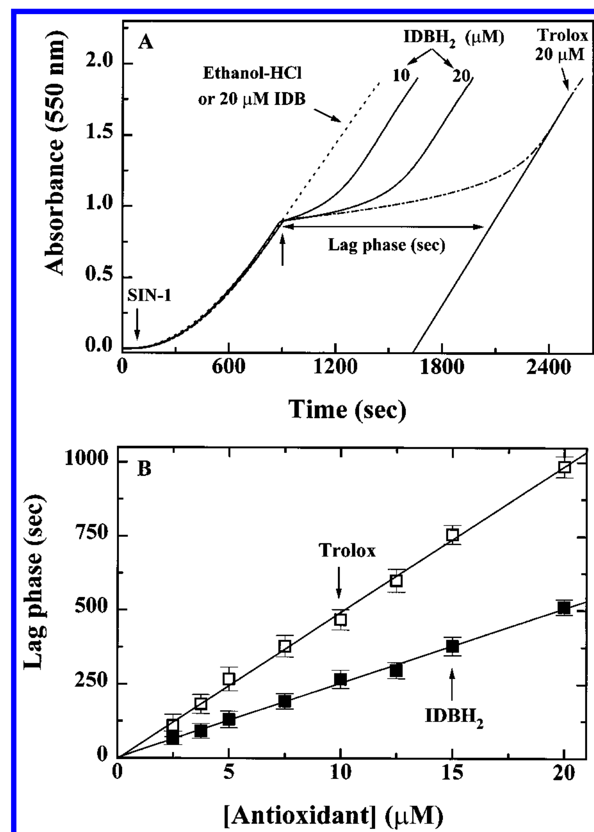


Figure 8. Effect of idebenone on ONOO$^-$-dependent oxidation of DHR. Incubations were prepared as described under Materials and Methods. Panel A shows the time-dependent oxidation of DHR by SIN-1 in the presence of 10−20 μM IDBH$_2$ (—) versus 20 μM Trolox (−·−) and 20 μM IDB or an equivalent volume of ethanol-HCl. In panel B the lag phases preceding the resumption of absorbance were plotted as a function of IDBH$_2$ (■) or Trolox (□) concentration.

hypervalent species of myoglobin before it could attack PnA. Once again, neither IDB nor ethanol-HCl could prevent the decay of PnA fluorescence.

Interaction of Idebenone with Peroxynitrite. Peroxynitrite is the product of the nearly diffusion-limited reaction between $O_2^{\bullet -}$ and $^{\bullet}$NO. Peroxynitrite is a powerful oxidant that damages many cellular components (*38, 39*) and experimental probes such as DHR (*28*). As shown in Figure 8A (dotted line), the oxidation of DHR by ONOO$^-$ was a time-dependent process that approached zeroth-order kinetics after ~10 min and proceeded for ~30 min. The addition of IDBH$_2$ 15 min after SIN-1 caused an instantaneous inhibition of DHR oxidation, which resumed after a lag phase that was strictly dependent on the antioxidant concentration (see also Figure 8A, solid line). DHR oxidation was similarly inhibited by Trolox, with the usual appearance of a lag phase that increased with increasing the concentration of the antioxidant (Figure 8). The slope values of the straight lines obtained by plotting lag phases versus antioxidant concentrations showed that IDBH$_2$ was 50% as efficient as Trolox in scavenging ONOO$^-$ (see also Figure 8B). IDB or an equivalent volume of ethanol-HCl did not inhibit DHR oxidation by ONOO$^-$ (see also Figure 8A).

Effects on ADP−Fe(II)-Dependent Microsomal Lipid Peroxidation and Cytochrome P450 Destruction. ADP−Fe(II) is known to promote lipid peroxidation in liposomal and microsomal systems (*40*). The precise mechanism of initiation remains unknown, but there is general agreement that ADP−Fe(II) must first oxidize with molecular oxygen (*40*). In our experiments, a chelate made of 500 μM ADP and 50 μM $FeSO_4$ was found to oxidize with oxygen at a linear rate of 2.432 ± 0.071 nmol/(mL·min)$^{-1}$, as measured by monitoring the disappearance of bathophenanthroline-chelatable Fe(II) in an inert medium like NaCl (*41*). This rate was not appreciably affected by 100 μM IDB [2.469 ± 0.038 nmol/(mL·min)$^{-1}$]; however, it was effectively decreased by equimolar IDBH$_2$ [0.108 ± 0.055 nmol/(mL·min)$^{-1}$]. Incubation of rat liver microsomes with ADP−Fe(II) resulted in extensive formation of TBARS, indicative of lipid peroxidation. This reaction was nearly abolished by IDBH$_2$ but not by IDB, in keeping with the different effects they had on ADP−Fe(II) oxidation (Table 2).

Lipid hydroperoxides generated upon ADP−iron-dependent initiation of peroxidative processes are known to react with cytochrome P450. This process accompanies destruction of the hemoprotein and liberates lipid alkoxyl radicals which reinitiate hydrogen abstraction from polyunsaturated fatty acid, thus propagating lipid peroxidation (*29, 42*). In this respect, Table 2 shows that (i) ADP−Fe(II)-dependent microsomal lipid peroxidation predictably caused the destruction of cytochrome P450 and (ii) the addition of IDBH$_2$ prevented cytochrome P450 destruction, although less effectively as compared with the concomitant inhibition of TBARS formation (75% vs 98%). The latter observation suggested that IDBH$_2$ uncoupled the destruction of cytochrome P450 from the propagation of lipid peroxidation. Inasmuch as propaga-

Table 2. Effects of Idebenone on ADP−Fe(II)- and CUOOH-Dependent Microsomal Lipid Peroxidation and Cytochrome P450 Destruction

system	TBARS (nmol/mg of protein)	P450 destruction (nmol/mg of protein)	TBARS/P450 (nmol/nmol)
ADP−Fe(II)[a]	21.03 ± 1.16	0.80 ± 0.09	26.28
+IDB	21.04 ± 1.34	0.74 ± 0.12	28.43
+$IDBH_2$	0.15 ± 0.05	0.21 ± 0.06	0.71
CUOOH[b]	7.40 ± 0.87	0.93 ± 0.11	7.96
+IDB	6.19 ± 0.50	0.54 ± 0.09	11.46
+$IDBH_2$	0.11 ± 0.08	0.32 ± 0.07	0.34

[a] Incubations were prepared and assayed for TBARS and cytochrome P450 destruction as described under Materials and Methods. Values are those determined after 5-min incubations. [b] Incubations were prepared and assayed as in footnote a with the exception that ADP−Fe(II) was replaced with CUOOH (0.25 mM).

tion accounts for ≥90% of TBARS formation (*42*), we could therefore calculate the ratio of TBARS to cytochrome P450 destruction as a tentative index of lipid radical reactions mediated by the hemoprotein. As also shown in Table 2, $IDBH_2$ decreased this ratio by greater than 95% in comparison with control incubations, confirming that $IDBH_2$ could interfere with the propagation of lipid peroxidation. On the other hand, IDB did not prevent cytochrome P450 destruction nor affect the ratio between TBARS accumulation and cytochrome P450 destruction.

The experiments described above suggested that $IDBH_2$ had the potential to inhibit lipid peroxidation not only by affecting ADP−iron reactions that initiate the peroxidative process but also by intercepting the propagating species generated upon subsequent involvement of cytochrome P450. To better characterize the latter possibility, we studied the effects of $IDBH_2$ on a lipid peroxidation system involving CUOOH in place of ADP−Fe(II). This system relies on the homolytic cleavage of CUOOH by cytochrome P450, yielding cumyl alkoxyl radicals that initiate lipid peroxidation (*43*). The reaction would subsequently proceed through the usual lipid hydroperoxide- and cytochrome P450-dependent mechanism, although both the exogenously added and endogenously formed hydroperoxide may contribute to the net destruction of the hemoprotein. As shown in Table 2, CUOOH induced lipid peroxidation and cytochrome P450 destruction. $IDBH_2$ prevented cytochrome P450 destruction by approximately 66%; however, some protection (42%) was also observed with IDB, perhaps reflecting bulky interferences with CUOOH−microsome interactions. Nevertheless, only $IDBH_2$ could significantly inhibit the formation of TBARS and the ratio with cytochrome P450 destruction. Collectively, these findings showed that (i) idebenone did interfere with cytochrome P450-dependent lipid peroxidation and (ii) the hydroquinone moiety was required to intercept lipid radicals formed upon cytochrome P450 cleavage of hydroperoxides and hemoprotein destruction.

Discussion

Drugs or natural compounds are very often studied in vitro to evaluate whether they can be used as antioxidants in vivo. Two major criteria should be kept in mind when performing this type of determination. First, test compounds should scavenge free radicals at concentrations attainable in tissues and biological fluids. Second, the free radical-scavenging efficiency of a given compound should stand comparison with other established antioxidants. According to such criteria, idebenone might be viewed as a first-choice drug for antioxidant interventions in free radical-mediated diseases. In fact, our studies demonstrate that idebenone can effectively scavenge a variety of free radical species, including DPPH and $ABTS^{\bullet+}$ (cf. Figure 1), peroxyl and tyrosyl radicals (cf. Figures 2−4), and $ONOO^-$ (cf. Figure 8). Idebenone can also redox couple with hypervalent species of Mb or Hb, thus preventing lipid peroxidation associated with the generation of these species (cf. Figures 5−7 and Table 1). In many cases, the antioxidant effects of idebenone become appreciable at ~2 μM, which is well in the range of plasma levels attainable in patients after oral doses of idebenone (*4*). When comparisons are made under appropriate conditions, the antioxidant efficiency of idebenone varies from no less than 50% to slightly more than 100% that of vitamin E or Trolox (cf. Figures 1−4 and 8). Furthermore, idebenone appears to protect biomolecules irrespective of whether they are placed in hydrophobic or hydrophilic environments. This is attested by experiments in which this compound could prevent peroxyl radical-dependent oxidation of water-soluble (*R*)-PE or liposome-incorporated PnA (cf. Figures 2 and 3). These results suggest that idebenone may intercept free radicals in both aqueous phases and lipid−water interfaces. Several reasonings and lines of evidence would therefore set the stage to conclude that idebenone is a broadly active and biologically relevant antioxidant.

Partially reduced species of dioxygen such as $O_2^{\bullet-}$ and H_2O_2 are currently given attention as possible mediators of neural dysfunctions, degenerative processes, or traumatic injury (*1, 2*). While not particularly reactive per se, both $O_2^{\bullet-}$ and H_2O_2 may become cytotoxic upon secondary reactions with other species. For example, $O_2^{\bullet-}$ may become toxic by reacting with $^{\bullet}NO$ and forming $ONOO^-$, which is currently believed to mediate neurotoxicity by excitatory amino acids such as glutamate (*39*). On the other hand, H_2O_2-dependent activation of Mb^{III} and Hb^{III} may very likely occur when vascular damage and bleeding set the premises for a leakage of these proteins within tissues, as in the case of brain trauma, stroke, or ischemia-reperfusion (*1*). Finally, H_2O_2 can activate myeloperoxidase to compound I and II intermediates that oxidize tyrosine to tyrosyl radicals (*44*). In terms of neuropathology, tyrosyl radicals might be formed and play some noxious role under conditions of ischemia-reperfusion and H_2O_2 formation by activated neutrophils and macrophages. In experiments monitoring the reduction of nitro blue tetrazolium by xanthine oxidase-generated $O_2^{\bullet-}$ or the stability of H_2O_2 at 240 nm, we could determine that idebenone lacks reactivity with either species (not shown). However, our data indicate that $IDBH_2$ readily interacts with $ONOO^-$, tyrosyl radicals, and H_2O_2-activated Mb^{III} or Hb^{III}. It follows that $IDBH_2$ can mitigate the toxicity of $O_2^{\bullet-}$ and H_2O_2 by scavenging those reactive species which would function as secondary and more potent mediators of oxidant damage.

One additional mechanism of toxicity by $O_2^{\bullet-}$ and H_2O_2 appears to involve the ability of these species to delocalize redox-active LMW iron from otherwise inactive cellular stores. Superoxide is known to release iron from ferritin (*45*) or enzymes endowed with Fe−S clusters (*46*).

Similarly, prolonged interactions of H_2O_2 with hemeproteins results in irreversible denaturation of the porphyrin pocket and consequent release of the iron coordinated therein (*47*). Once delocalized in a LMW form, iron promotes lipid peroxidation through reaction mechanism(s) that may not depend any longer on $O_2^{\bullet-}$ and H_2O_2. This is the case for ADP−Fe(II) complexes which initiate liposomal or microsomal lipid peroxidation by virtue of SOD- and catalase-insensitive mechanisms (*48*). ADP−iron-dependent lipid peroxidation probably proceeds through the oxidation of Fe(II) with oxygen and the formation of perferryl species [Fe(II)O_2−Fe(III)$O_2^{\bullet-}$] or poorly characterized Fe(II)−O_2−Fe(III) complexes that abstract hydrogen from the bis-allylic bonds of polyunsaturated fatty acids (*40, 49*). In either case, lipid peroxidation best occurs when appropriate Fe(II):Fe(III) ratios are formed, although the molecular basis for such a requirement has remained a matter of debate (*40*). Our results demonstrate that idebenone affects the oxidation of ADP−Fe(II) with oxygen and thus precludes the initiation of lipid peroxidation by reactive species which require some Fe(III) (cf. Table 2). Simultaneous determinations of cytochrome P450 destruction and comparative experiments in CUOOH-dependent systems suggest that idebenone may also scavenge lipid-reactive species which propagate lipid peroxidation and concur in microsomal damage (Table 2). Idebenone can therefore mitigate the toxicity associated with iron delocalization and oxidative deterioration of biological membranes.

Previous studies on the antioxidant functions of idebenone have not conclusively established whether it acts in the hydroquinone or quinone form (*7−12*). The experiments described in this paper would suggest that the antioxidant effects of idebenone pertain exclusively to its hydroquinone form, $IDBH_2$; the quinone form, IDB, cannot scavenge $ABTS^{\bullet+}$, peroxyl, or tyrosyl radicals, nor can it react with $ONOO^-$- or H_2O_2-activated hemoproteins. Likewise, IDB does not interfere with ADP−iron- or CUOOH-dependent microsomal lipid peroxidation. The experiments with ferrylmyoglobin also demonstrate that reactions between $IDBH_2$ and excited species accompany its stoichiometric conversion to IDB. The antioxidant effects of idebenone are therefore mediated by electron-transfer mechanisms that involve oxidation of $IDBH_2$ to IDB. Similar mechanisms have been described for CoQ_{10} and imply that antioxidant interventions with idebenone become effective following the reduction of IDB to $IDBH_2$. Unfortunately, site(s) and mechanism(s) of IDB reduction are unknown. In principle, IDB might be reduced by the same mitochondrial dehydrogenases that reduce CoQ_{10}; however, recent studies have shown that plasma membranes from various cell types, including erythrocytes and hepatocytes, contain NAD(P)H dehydrogenases which reduce more rapidly short-chain coenzyme Q analogues than CoQ_{10} (*50, 51*). This alternative pathway of reduction has also been described with purified DT-diaphorase (*52*). As mentioned, the structure of idebenone is strongly reminiscent of short-chain CoQ analogues; hence, both CoQ_{10} and CoQ_1 reductases might concur in converting idebenone from IDB to $IDBH_2$, making it suitable for redox coupling with noxious radicals.

Antioxidant interventions with vitamin E or CoQ_{10} are limited by pharmacokinetic constraints. Coenzyme Q_{10} approaches high plasma levels but enters tissues rather poorly (*53, 54*). It follows that CoQ_{10} supplementation may be a good strategy to ameliorate oxidant damage within the plasma milieu (e.g., LDL oxidation) but not in intracellular environments. Likewise, vitamin E penetrates the central nervous system slowly and may thus fail to afford protection in neural cells acutely exposed to oxidative injury (*55*). The clinical usefulness of vitamin E might therefore be confined to the long-term supplementation of patients affected by chronic degenerative processes, such as Alzheimer's disease (*56*). These problems are not observed with idebenone which distributes throughout the body and readily approaches similar concentrations in tissues and fluids (*4, 57, 58*). These pharmacokinetic properties, as well as the antioxidant functions described in our study, may help clarify the neuroprotective action of idebenone and its greater efficiency in comparison with vitamin E or CoQ_{10} (*9, 12*).

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