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Superoxide Dismutase Enhances Chain-Breaking Antioxidant Capability of Hydroquinones

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2-tert-butyl- (**1**), 2,6-dimethyl- (**2**), 2,5-dimethyl- (**3**), trimethyl- (**4**), and 2,3-dimethoxy-5-methyl- (**5**) substituted p-hydroquinones (QH_2) were tested as a chain-breaking antioxidant during the oxidation of methyl linoleate (ML) in dodecyl sulfate micellar solution, pH 7.40, at 37 °C. In the absence of superoxide dismutase (SOD), all the studied QH_2 displayed very moderate if any antioxidant capability. When 5–25 U/ml SOD was added, QH_2 showed a pronounced ability to inhibit ML oxidation. The stoichiometric factor of inhibition was found to be about one for all the tested QH_2 in the presence of SOD. The reactivities of QH_2 to the ML peroxy radical increase in the order QH_2 **5** < QH_2 **3** < QH_2 **1** ≈ QH_2 **2** < QH_2 **4**; reactivity of QH_2 **4** exceeds that reported for the majority of phenolic antioxidants. The features of QH_2 as an antioxidant in aqueous environment is likely associated with the reactivity of semiquinone ($O^{\bullet -}$) formed due to attack of the peroxy radical to QH_2. $O^{\bullet -}$ reacts readily with molecular oxygen with formation of superoxide ($O_2^{\bullet -}$); in turn, $O_2^{\bullet -}$ attacks both to QH_2 and ML (likely, as $HO_2^{\bullet}$) that results in fast depleting QH_2 and chain propagation, respectively. The addition of SOD results in purging a reaction mixture from $O_2^{\bullet -}$ and, as a corollary, in depressing undesirable reactions with the participation of $O_2^{\bullet -}$. Under these conditions, QH_2 displays the theoretically highest inhibitory activity which is determined solely by the reactivity of QH_2 to the peroxy radical.

Keywords: Chain-breaking antioxidant, lipid peroxidation, inhibition, hydroquinones, superoxide dismutase, methyl linoleate

Abbreviations: QH_2, hydroquinone; $O^{\bullet -}$, semiquinone; Q, quinone; $QH^{\bullet}$, phenoxy radical; $LO_2^{\bullet}$, peroxy radical; $O_2^{\bullet -}$, superoxide; SOD, superoxide dismutase; ML, methyl linoleate; AAPH, 2,2′-azobis(2-amidinopropan) dihydrochloride; SDS, sodium dodecyl sulfate; HPMC, 6-hydroxy-2,2,5,7,8-pentamethylbenzochroman; R, rate of inhibited oxidation; R_0, rate of non-inhibited oxidation; R_{IN}, rate of initiation; t_{IND}, induction period

INTRODUCTION

Hydroquinones (QH_2) are rather abundant in biological systems, commonly coexisting with corresponding quinones (Q). QH_2 may serve as both a pro-oxidant and antioxidant. The pro-oxidant role and the cytotoxicity of QH_2 are associated with their tendency to be oxidized by molecular oxygen with formation of active free radicals. Chain-breaking antioxidative activity of QH_2 may be due to reaction of QH_2 with

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peroxyl radicals ($LO_2^{\bullet}$)

$$(1)\quad QH_2 + LO_2^{\bullet} \rightarrow QH^{\bullet} + LOOH \qquad k_1$$

While the kinetics of QH_2 autoxidation have been studied in many details,[1–3] and the interrelation between QH_2 structure and their oxidizability under biological environment has been recently well established,[3] only rather poor information on chain-breaking antioxidative activity of QH_2 is available in the literature. High reactivity of QH_2 to $LO_2^{\bullet}$ is expected from their structure[4] and well documented for a few QH_2 during the oxidation of styrene.[5–6] As for the inhibition of lipid peroxidation by QH_2 in microheterogeneous lipid-aqueous systems (liposomes, micelles), this has been determined only for some ubiquinols,[7,8] for α-tocopheryl hydroquinone[8] and for di-tert-butylcatechol.[6]

In this work, chain-breaking antioxidant capabilities of several substituted 1,4-hydroquinones were determined during the oxidation of methyl linoleate (ML) in micellar solution of sodium dodecyl sulfate (SDS). This kinetic model has been widely used,[9–11] along with liposomes, to determine chain-breaking antioxidant activity of various substances under the conditions to some extent imitating lipid peroxidation in biological systems. Compared to liposomal models, the micellar ones have the advantage that diffusion limitations for Reaction (1) are much less pronounced.[11,12]

MATERIALS AND METHODS

Trimethyl-1,4-hydroquinone was purchased from Aldrich; all the other hydroquinones were prepared from the corresponding quinones. 2,6-Dimethyl-1,4-benzoquinone was purchased from Aldrich; 2,3-dimethoxy-5-methyl-1,4-benzoquinone from Sigma; 2,5-dimethyl-1,4-benzoquinone was obtained from Fluka; tert-butyl-1, 4-benzoquinone from EGA Chemie. Quinones were converted into hydroquinones by reduction with sodium tetrahydroborate in tetrahydrofurane and then purified by recrystallization from an appropriate solvent or using silica gel (40–100 μm) column with $CHCl_3$ as an eluent. Methyl linoleate (ML) as well as superoxide dismutase from bovine erythrocytes with the activity of 4000–7000 U/mg were purchased from Sigma. Sodium phosphates, NaH_2PO_4 and Na_2HPO_4, of the highest grade used to prepare buffer solution as well as sodium dodecyl sulfate, were purchased from Merck. 2,2′-azobis (2-amidinopropan) dihydrochloride (AAPH) was obtained from Polysciences, Inc.

Aqueous solutions were prepared with doubly distilled water. Experiments were performed at 37.0 ± 0.1 °C with 50 mM phosphate buffer, pH 7.40 ± 0.02, which was prepared by mixing 50 mM solutions of NaH_2PO_4 and Na_2HPO_4 without adding any acid or base. Solutions of the individual phosphates used for the buffer preparation were purged from traces of transition metals by Chelex-100 resin (Bio-Rad) using a batch method. Stock solutions of QH_2 were prepared with acid DMSO typically under argon atmosphere and stored no longer than one day at −25 °C.

The kinetics of oxygen consumption were studied with a 5300 Oxygen Biological Monitor (Yellow Springs Instruments Co., USA) computerized by using a ME-32 Multimeter (Metex, Korea) as an interface and the Micrisoft program Origin to treat data. The order of reagent additions was as follows: SDS in buffer, AAPH, SOD if required, ML. Runs were started with the determination of the rate of non-inhibited oxidation (R_0) and the rate of initiation (R_{IN}) (see below). Then QH_2 was added as a stock solution. The protocol for preparation of solutions and kinetic studies of oxygen consumption has been given in more detail in our previous publications.[3,11,13–15]

Inhibitory activity of QH_2 was characterized by two parameters – the rate constant for reaction (1), k_1, and stoichiometric factor of inhibition, f, which shows how many kinetic chains can be terminated by one molecule of antioxidant. A value of k_1 was determined through the

competition for $LO_2^{\bullet}$ between Reaction (1) and chain propagation

$$(2)\quad LO_2^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \qquad k_2$$

under conditions of steady generation of active free radicals. The latter was achieved thanks to the thermodecomposition of water-soluble azo-compound AAPH

$$(3)\quad AAPH + (LH + O_2) \longrightarrow LO_2^{\bullet} + \text{products} \quad k_{IN};\quad R_{IN} = k_{IN} \cdot [AAPH]$$

The rate of free radical generation (initiation), R_{IN}, was determined from the induction period, t_{IND}, during the oxidation in the presence of a reference antioxidant, 6-hydroxy-2,2,5,7,8-pentamethylbenzochroman (HPMC), for which f was reported to be as much as 2.[4,5] R_{IN} was calculated from t_{IND} using the equation

$$R_{IN} = f \cdot [HPMC]/t_{IND} \qquad (1)$$

This approach may be also applied to determine f for QH_2

$$f = t_{IND} \cdot R_{IN}/[QH_2] \qquad (2)$$

However, for antioxidants with relatively low value of k_1, a common graphic procedure to determine t_{IND} (Figure 1), which actually has no reliable theoretical basis, is not correct. In this case, t_{IND} may be more correctly determined by integration (Figure 2) where R and R_0 are the rate of chain oxidation in the presence and in the absence of antioxidant, t_{IND}

$$t_{IND} = \int_{\infty}^{0} (1 - R/R_0)dt \qquad (3)$$

respectively. With more active antioxidants, both methods of determining t_{IND} give practically the same values of t_{IND} and f (not shown).

Contrary to the oxidation of lipids in homogeneous solutions, where the rate of chain oxidation is proportional to square root of R_{IN}, this rate in SDS micelles is nearly directly with R_{IN}.[11,15] The latter means that the chain termination occurs basically by a reaction of first order in $[LO_2^{\bullet}]$ rather than via common bimolecular reaction

$$LO_2^{\bullet} + LO_2^{\bullet} \longrightarrow \text{products}$$

For this case, the expression relating k_1/k_2 and the change in R with time is given by the equation

$$F = \ln\left(\frac{R_0}{R} - 1\right) + \frac{R_0}{R} = -\frac{k_1 R_0}{k_2[LH]}t + \text{const} \qquad (4)$$

where [LH] is the concentration of oxidation substrate (ML in our case). The deduction of

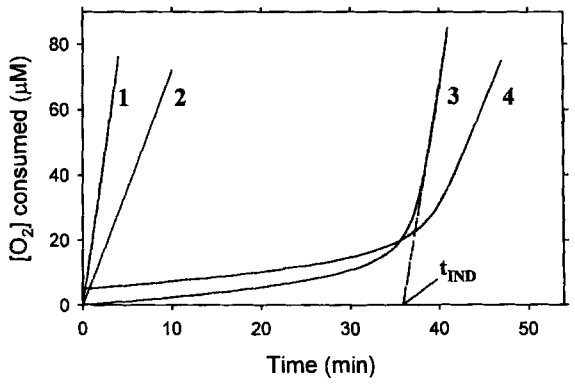

FIGURE 1 $[O_2]$ traces recorded during the oxidation of 20 mM ML in micellar solution of SDS in the absence of SOD (plots 1 and 3) and with 20 U/ml SOD added (plots 2 and 4). 1 and 2 – the oxidation without antioxidant, 3 and 4 in the presence of 3 μM HPMC (for plot 4 y-axis is shifted by 5 μM O_2). Conditions: [AAPH] = 5 mM; [SDS] = 0.2 M; 50 mM sodium phosphate buffer, pH 7.40; 37 °C.

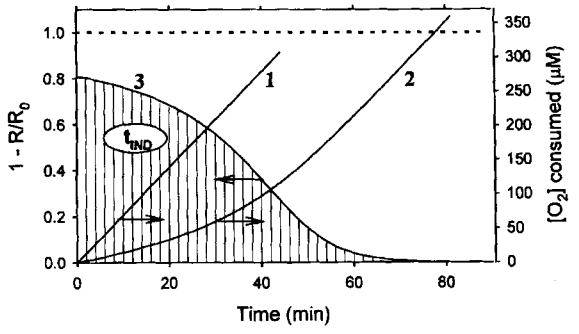

FIGURE 2 Determination of t_{IND} from $[O_2]$ trace observed during the oxidation of 20 mM ML in SDS micellar solution inhibited by QH_2 5: plot 1 – $[O_2]$ trace recorded during the oxidation without antioxidant;. plot 2 – $[O_2]$ trace recorded during the oxidation in the presence of 5 μM QH2 5; plot 3 – kinetics of the change of $(1 - R/R_0)$ value with time calculated on the base of plot 2. The cross-hatched area corresponds to t_{IND} (see Equation 3). Conditions: [AAPH] = 5 mM; [SDS] = 0.2 M; 50 mM sodium phosphate buffer, pH 7.40; 37 °C.

Equation 4 has been given in our previous works.[11,15] It may be pointed out that the application of Equation 4 does not require the knowledge of antioxidant concentration.

RESULTS

The addition of even moderate concentration of SOD resulted in the substantial decrease in R_0 (Figure 1). This suggests the formation of $O_2^{\bullet-}$ during the oxidation of ML and its participation, likely as $HO_2^{\bullet}$, in chain propagation. Most likely, the formation of $HO_2^{\bullet}$ ($O_2^{\bullet-}$) occurs thanks to β-decay of $LO_2^{\bullet}$ as this has been previously predicted theoretically on the base of thermodynamic estimations.[16] Essentially, SOD did not affect R_{IN}; the value of k_{IN} (calculated as $R_{IN}/[AAPH]$) was found to be as much as $(5.8 \pm 0.3) \times 10^{-7}\,s^{-1}$ (averaged from ca. 30 separate measurements conducted at [SOD] from 0 to 50 U/ml). SOD effect on lipid peroxidation in micellar solutions will be published in more detail elsewhere. In the absence of SOD, all the tested hydroquinones displayed very moderate if any inhibiting activity (Figure 3A). Even the starting rate of oxygen consumption measured immediately after the addition of QH_2 was only slightly lower than R_0 and rapidly increased with time arriving at R_0 in a few minutes (Figure 3A). An effective value of f calculated from $[O_2]$ traces did not exceed 0.1.

When 5–25 U/ml SOD was added, chain-breaking activity of QH_2 increased dramatically and a pronounced inhibition for an extended period of time was observed (Figure 3B). At $[QH_2] \geq 10\,\mu M$, a starting rate of oxidation dropped to a value which is not too different from the rate of non-chain oxidation which, in turn, is equal to $2R_{IN}$. In the case of the most active antioxidant, QH_2 **4**, a pronounced induction period was observed (Figure 3B). With all the tested QH_2, the duration of induction period was proportional to $[QH_2]$ as it is exemplified by Figure 4. For all QH_2 tested, parameter f was found to be ca. one (Table I). Nearly the same kinetic regularities were observed when these experiments were conducted with micelles of Triton-100X instead of SDS (not shown). This testifies that the behavior of p-QH_2 as an antioxidant is not associated with a nature of SDS micelles. Meanwhile, the inhibitory activity of

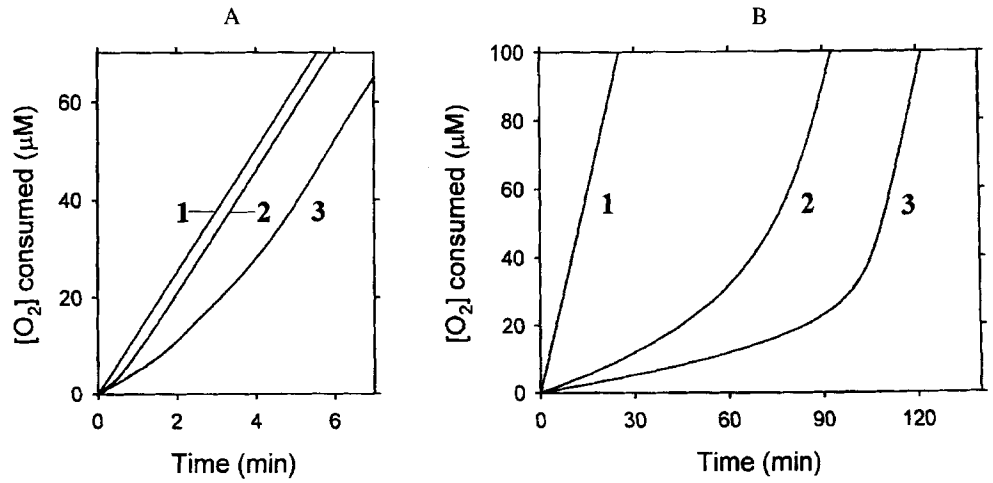

FIGURE 3 $[O_2]$ traces recorded during the oxidation of 20 mM ML in micellar solution of SDS in the absence of SOD (plot **A**) and with 20 U/ml SOD added (plot **B**). Traces 1 – without antioxidant; traces 2 – with 7 μM QH_2 **2**; traces 3 – with 10 μM QH_2 **4**. Conditions: [AAPH] = 3 mM; [SDS] = 0.2 M; 50 mM sodium phosphate buffer, pH 7.40; 37 °C.

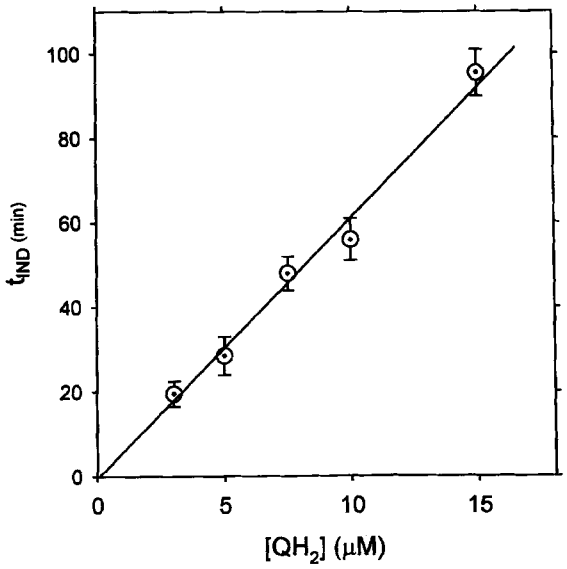

FIGURE 4 Plot of the induction period against [QH_2] observed during the oxidation of 20 mM ML in micellar solution of SDS with 20 U/ml SOD added; inhibitor is QH_2 4. Conditions: [AAPH] = 5 mM; [SDS] = 0.2 M; 50 mM sodium phosphate buffer, pH 7.40; 37 °C.

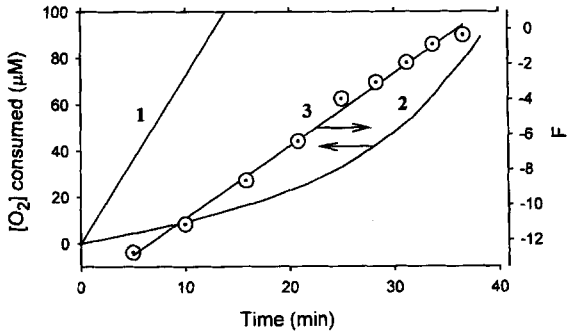

FIGURE 5 Kinetics of the oxidation of 20 mM ML in micellar solution of SDS with 20 U/ml SOD added in the presence QH_2 5. 1 – [O_2] trace without antioxidant; 2 – [O_2] trace with added 3 μM QH_2 2; 3 – trace 2 in the axes of Equation 4. Conditions: [AAPH] = 5 mM; [SDS] = 0.2 M; 50 mM sodium phosphate buffer, pH 7.40; 37 °C.

HPMC (Figure 1, plots 3 and 4) and Trolox (not shown) was found to be almost unaffected by SOD.

Figure 5 gives an example of k_1/k_2 determination in the presence of SOD. It can be seen that a [O_2] trace gives a straight line in the axes of Equation 4. A value of k_1/k_2 may be determined from the slope of a line using Equation 4. The values of k_1/k_2 determined by such a way are given in Table I. The rate constant k_2 required to calculate absolute values of k_1 from k_1/k_2, was assumed to be of 70 $M^{-1}s^{-1}$ (average value from those reported in several works).[3,12] The comparison of k_1/k_2 for QH_2 with those for monophenolic antioxidants tested also in SDS micelles[9–11] shows that alkyl- and methoxy-substituted QH_2 display rather high reactivity towards $LO_2^{\bullet}$. For the most active QH_2 **4**, k_1/k_2 exceeds this value for all the studied monophenolic antioxidants, both synthetic and natural, with a few exceptions of HPMC (4100–4400),[10,11] Trolox (1600–3000)[8–10] and some other synthetic analogues of vitamin E.[8] For two antioxidants, QH_2 **4** and QH_2 **5**, k_1 has been earlier reported during the oxidation of homogeneous styrene to be as much as 1.4×10^6 and 3.1×10^5 $M^{-1}s^{-1}$, respectively.[5] Comparison of the above values with those determined in this study (Table I) shows that going from the oxidation of a homogeneous hydrocarbon to the oxidation of ML in micelles is accompanied by the decrease in k_1 by nearly one order of magnitude.

TABLE I Chain-breaking antioxidant activity of substituted 1,4-hydroquinines during the oxidation of 20 mM ML in the presence of 20 U/ml SOD in 0.2 M SDS micelles in 50 mM sodium phosphate buffer, pH 7.40, at 37 °C

QH_2	Substituents	f[a]	k_1/k_2[a]	$k_1 \times 10^{-4}$ ($M^{-1}s^{-1}$)[b]
1	tert-Bu-	0.85 ± 0.07	1100 ± 120	7.7
2	2,6-Me_2-	0.99 ± 0.04	1080 ± 80	7.6
3	2,5-Me_2-	1.04 ± 0.03	860 ± 30	6.0
4	Me_3-	1.00 ± 0.06	2820 ± 340	19.7
5	2,3-$(MeO)_2$-5-Me-	0.92 ± 0.08	470 ± 40	3.3

[a]Averaged from at least three experiments.
[b]Calculated from k_1/k_2 assuming that k_2 is equal to 70 $M^{-1}s^{-1}$ (see text).

The related effect has been earlier reported for many phenolic antioxidants.[6,8–10,14] The most likely reason for the decrease in k_1 during the oxidation of lipids in micellar solutions is the formation of H-bonds between OH-group possessed by phenols and molecules of water[6,9–11,15] as well as carboxyl group associated with fatty ester.[11,17]

DISCUSSION

As indicated above, p-OH_2 and monophenolic compounds display a visible difference in their behavior as a chain-breaking antioxidant. Whereas monophenolics provide a pronounced inhibition of lipid peroxidation, which is not affected by SOD, with f being commonly about two per one OH-group, p-QH_2 show a pronounced chain-breaking capability in the presence of SOD only with f as much as one (Table I). The features of OH_2 as an antioxidant cannot be explained by the inclination of QH_2 to the oxidation by molecular oxygen.[3] While SOD enhances the inhibitory capability of all the tested QH_2, the addition of SOD has been reported to accelerate the oxidation of QH_2 **2** and **3**, but has only a moderate effect on the oxidation of QH_2 **4** and **5**.[3] Thus, there is no correlation between the influence of SOD on the rate of QH_2 autoxidation and the ability of QH_2 to inhibit lipid peroxidation. As for the consumption of QH_2 **1–4** due to autoxidation, the rate of this process was reported to be too low to provide any substantial consumption within a few minutes when some inhibition is observed. In particular, QH_2 **1–4** oxidize at the rate of ca. 0.5%/min;[3] it means that not more than a few percent of QH_2 is expected to be consumed during the period of time, for which the inhibitory action of QH_2 comes to the end (see Figure 3A).

Most likely, the difference in the behavior of p-OH_2 and monophenolic antioxidants is due to the difference in reactivity between free radicals $QH^\bullet$ and $PhO^\bullet$ formed by the reaction of $LO_2^\bullet$ with p-OH_2 and monophenolic antioxidants, respectively. $PhO^\bullet$ is known as a rather non-reactive species for the majority of kinetic situations and is commonly terminated by reaction with $LO_2^\bullet$

$$(4)\quad PhO^\bullet + LO_2^\bullet \longrightarrow \text{products}$$

The sequence of Reactions (1) and (4) provides $f = 2$. Meanwhile, $QH^\bullet$, having, as a rule, pK less than 4–5,[18] undergoes fast deprotonation at neutral pH

$$(5)\quad QH^\bullet \longrightarrow Q^{\bullet-} + H^+$$

In turn, semiquinone, $Q^{\bullet-}$, reacts readily with molecular oxygen

$$(6)\quad Q^{\bullet-} + O_2 \longrightarrow O_2^{\bullet-} + Q \qquad k_6$$

For instance, k_6 was reported (in $M^{-1}s^{-1}$) to be as much as 1.1×10^6,[19] 8.8×10^6, 3.4×10^6[18] for QH_2 **1**, **2** and **3**, respectively. Meanwhile, k_6 is known to increase when one-electron-reduction potential $E(Q/Q^{\bullet-})$ becomes more negative.[20] For QH_2 **4** ($E(Q/Q^{\bullet-}) = -165\,mV$)[21] and QH_2 **5** ($E(Q/Q^{\bullet-}) = -110\,mV$)[22] k_6 is expected to exceed $1 \times 10^8\,M^{-1}s^{-1}$. Under kinetic conditions typical of our study, Reaction (6) is expected to be the main path of $Q^{\bullet-}$ transformation for all the studied OH_2. In turn, $O_2^{\bullet-}$ formed through Reaction (6) attacks to QH_2[23,24]

$$(7)\quad O_2^{\bullet-} + QH_2 \longrightarrow H_2O_2 + Q^{\bullet-} \qquad k_7$$

and LH (likely in the protonated form)

$$(8)\quad HO_2^\bullet + LH(O_2) \longrightarrow H_2O_2 + LO_2^\bullet \qquad k_8$$

Both Reactions (7) and (8) result in the decrease in inhibitory capability of QH_2. Owing to Reaction (7), antioxidant is rapidly depleted, which results in the decrease of f. Reaction (8) is conceptually a chain-propagating reaction and its contribution increases the rate of the inhibited oxidation.

The addition of SOD results in purging a reaction mixture from $O_2^{\bullet-}$

$$(9)\quad O_2^{\bullet-}(+O_2^{\bullet-}) + SOD + 2H^+ \longrightarrow O_2 + H_2O_2 + (SOD) \qquad k_9$$

and, as a corollary, in depressing Reactions (7) and (8). If the concentration of added SOD is

high enough, the contributions of Reactions (7) and (8) can be neglected, and the chain-breaking capability of QH_2 arrives at a theoretically maximal level, when the extend of inhibition is only determined by the parameter k_1/k_2. In this case the stoichiometric factor f is as much as one.

A maximal value of f for all the tested QH_2 was practically achieved even at SOD concentration less than 20 U/ml (not shown). Unfortunately, the study of concentration effect of SOD on f in more detail is complicated as this effect is superimposed by the effect of SOD on the rate of non-inhibited oxidation of ML (see above). Nonetheless, SOD concentration required to provide the limited inhibitory activity of QH_2 ([SOD]*) may be estimated from the competition for $O_2^{\bullet-}$ between Reactions (9) and (7) or (8). The rate of Reaction (9) will exceed the rate of Reaction (7) under condition that

$$[SOD]^* > k_7[QH_2]/k_9 \quad (5)$$

The same condition for Reaction (8) is

$$[SOD]^* > k_8[LH]/k_9 \quad (5a)$$

The rate constant for Reaction (9) at neutral pH was reported to be ca. $2 \times 10^9\,M^{-1}s^{-1}$.[23,24] The information on k_7 in the literature is rather poor and conflicting; a typical value of k_7 is $1 \times 10^5\,M^{-1}s^{-1}$[23,24] that is less than k_9 by four orders of magnitude. The latter means that the condition (5) will be met if $[SOD]^*/[QH_2] > 5 \times 10^{-5}$. Taking $[QH_2]$ to be as much as 10 μM, [SOD]* is predicted to be ca. 5×10^{-10} M (about 5 U/ml), which is much less than a common physiological concentration of SOD in tissues (1–10 μM).[25] A value of k_8 is likely not far from $k_2 = 70\,M^{-1}\,s^{-1}$ and [SOD]* estimated from relation (5a) hardly exceeds the previous value of [SOD]*. We can conclude from the above estimations that QH_2 display a maximal chain-breaking capability under the majority of *in vivo* conditions.

Admittedly, the above discussed kinetic picture is likely somewhat simplified and is not absolutely general. In particular, if we deal with more lipophylic QH_2, a significant portion of free radical $QH^\bullet$ is expected to occur in lipid phase and does not undergo deprotonation. QH_2 of such a kind likely display a pronounced inhibiting activity even in the absence of SOD. It is not excluded that rather high lipophylity of ubiquinols[7] and di-tert-butylcatechol[6] is a reason for their significant antioxidant activity in the absence of SOD. It is possible to discuss with work[8], where a very moderate effect of UQH2 and TQH2 during the oxidation of ML in SDS micelles was also observed. However, SOD effect was not studied in work[8] and the other reason for low capability of UQH2 and TQH2 (long tail!) may be suggested. The number of QH_2 studied is evidently not enough to make inferences about the relationship of antioxidant activity of QH_2 with their structure, and further experiments are required.

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