Free Radical Research, Volume 38 Number 10 (October 2004), pp. 1107–1112

Taylor & Francis
healthsciences

Quinone-enhanced Ascorbate Reduction of Nitric Oxide: Role of Quinone Redox Potential

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Accepted by Professor V. Darley-Usmar

(Received 7 July 2004; In revised form 27 August 2004)

The quinones 1,4-naphthoquinone (NQ), methyl-1,4-naphthoquinone (MNQ), trimethyl-1,4-benzoquinone (TMQ) and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ-0) enhance the rate of nitric oxide (NO) reduction by ascorbate in nitrogen-saturated phosphate buffer (pH 7.4). The observed rate constants for this reaction were determined to be 16 ± 2,215 ± 6,290 ± 14 and $462 \pm 18\,M^{-1}\,s^{-1}$, for MNQ, TMQ, NQ and UQ-0, respectively. These rate constants increase with an increase in quinone one-electron redox potential at neutral pH, E_7^1. Since NO production is enhanced under hypoxia and under certain pathological conditions, the observations obtained in this work are very relevant to such conditions.

Keywords: Quinones, 1,4-naphthoquinone; Nitric oxide; Methyl-1,4-naphthoquinone; Trimethyl-1,4-benzoquinone; 2,3-dimethoxy-5-methal-1,4-benzoquinone

INTRODUCTION

Quinones are widely distributed compounds in nature. These are found in humans as endogenous compounds, such as coenzyme Q, the metabolites of estrogen hormones,[1] or as exogenous compounds, such as those used clinically[2], or those produced as metabolites of polycyclic aromatic compounds.[3] Due to the importance of quinones in humans, and the high reactivity of quinones, substantial research on the chemistry and toxicology of these compounds has taken place.

One of the toxic capabilities of quinones is that these accelerate the oxidation of ascorbate.[4–12] For quinones with redox potentials below -100 mV, it has been proposed that the mechanism for ascorbate (AH^-) oxidation follows two elementary steps, being the quinone reduction step rate-limiting.[4] Equations (1) and (2), depict these elementary reactions, where Q and $Q^{\cdot-}$ are the quinone and the semiquinone,

$$Q + AH^- \rightarrow Q^{\cdot-} + A^{\cdot-} + H^+ \quad (1)$$

$$Q^{\cdot-} + O_2 \rightarrow Q + O_2^{\cdot-} \quad (2)$$

respectively. Since reaction (1) is rate-limiting, it is found that the "effective" rate constants (which are essentially the rate constants for reaction (1)) increase with an increase in quinone redox potential. In this work, we report a similar behavior for the anaerobic ascorbate oxidation in the presence of quinones and nitric oxide (NO).

Nitric oxide is a free radical formed in a variety of cell types by NO synthase and has many important physiological roles such as acting as vasorelaxant,[13] a neurotransmitter[14,15] and performing other physiological and pathophysiological phenomena.[16] The one-electron reduced derivative of NO, the nitroxyl anion, $^3NO^-$ (or its conjugate acid, ^1HNO), has been postulated to be a cytotoxic species *in vitro*.[17,18] Due to the large p*K*a value of ^1HNO, ca. 11.6, the conjugated acid, ^1HNO, is the only significant reduced NO species under physiological conditions.[19,20] This species has also been postulated to promote tissue injury in hypoxic processes, such as ischemia-reperfusion, cancer and stroke.[21–23] The combination of ^1HNO plus oxygen has been shown to be more cytotoxic than ^1HNO alone.[24] An oxidized intermediate formed by the reaction of ^1HNO and oxygen has been proposed as

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400009852

the cytotoxic species.[17] Furthermore, NO production is enhanced under hypoxic conditions in several tissues.[25–27] Thus, if the rate of nitric oxide reduction to form the nitroxyl species is enhanced by quinones under anaerobic or hypoxic conditions, ^{1}HNO toxicity should also be enhanced by quinones when the tissue is exposed to air.

It is the goal of this work to study the role of quinones on the ascorbate reduction rate of nitric oxide under anoxic conditions. We have measured here the rate of NO consumption by ascorbate in the presence and absence of quinones.

MATERIALS AND METHODS

Chemicals

The quinones (Fig. 1) 1,4-naphthoquinone (NQ), methyl-1,4-naphthoquinone (MNQ) and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ-0) were purchased from Aldrich Chemical Co. The hydroquinone of trimethyl-1,4-benzoquinone (TMQ) was obtained from Alfa Aesar and oxidized to the quinone using AgO as described elsewhere.[28] Quinones were purified by double sublimation before used. Diethylamine NONOate (DEA/NO) was purchased from Calbiochem and stored under dry ice. Nitrous oxide was obtained from Puritan Medical Products. Only distilled deionized and Chelex- treated water was used in this work. Chelex treatment of water was monitored using the ascorbate test, as described by Buettner.[29]

Nitric Oxide Reduction Kinetics

These were monitored using a NO-specific electrochemical probe (ISO-NOP) inserted in a thermostated NO chamber (World Precision Instruments, Sarasota, FL, USA) at 37°C. A weighed amount (using a Cahn C-23 micro balance) of DEA/NO was added to the NO chamber in order to produce a 10 μM NO solution after adding all reagents. The chamber was purged with high purity nitrogen followed by immediate injection of 1.00 ml of a nitrogen-saturated solution containing 50 mM phosphate buffer (pH 7.4), 100 μM DETAPAC and from 0 to 80 μM quinone. Care was taken to immediately exclude all gas bubbles out of the sample, through the chamber capillary, after DEA/NO contacting this aqueous solution, in order to avoid NO partition into the gas phase. The last reagent added, after maximum DEA/NO decomposition was reached, was a nitrogen-saturated ascorbate solution with final concentrations in the sample of 0–80 μM. Basal voltage was calibrated to zero every day. Voltage output corresponding to a 10 uM NO solution was checked every day and the electrode membrane was replaced in case there was not agreement with previous outputs within 10%. The electrode was calibrated with known concentrations of $NaNO_2$ by reacting this salt with KI in sulfuric acid medium. NO consumption data were collected in a computer and the initial rates of NO consumption ($R_{NO,0}$) were measured.

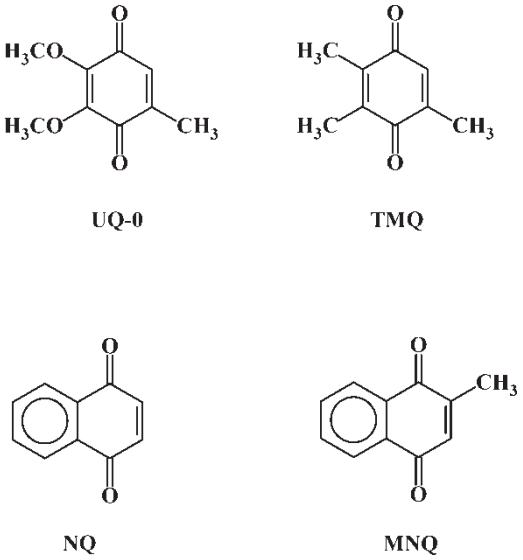

FIGURE 1 Quinones under study here.

Nitrous Oxide Determination

Exactly 0.8 mg of DEA/NO was weighed in a Cahn micro balance and placed in a septum-stoppered bottle followed by purging with high purity N_2. Aliquots of solutions containing quinone, DETAPAC and phosphate buffer (pH 7.4) were then added to this bottle. After 15 min, an aliquot of ascorbate in water was then added. Initial reagent concentrations in the sample were 50 mM phosphate buffer, 100 μM DETAPAC, 100 μM quinone and 2 mM ascorbate. A sample of the gaseous phase was withdrawn from the reaction vessel and injected in the chromatograph after 30 min of adding ascorbate to the sample. Nitrous oxide was detected using an Agilent 6890 gas chromatograph equipped with a Porapak Q column ($6' \times 1/8''$) and a thermal conductivity detector operating at 40°C with a flow rate of 7 ml/min. The N_2O peak was identified using a standard of N_2O from a lecture bottle.

In order to determine the mol ratio of N_2O produced to that of NO consumed, the amount of NO consumed was measured in the NO chamber at 37°C by following NO consumption in a 1.00 ml anaerobic sample containing 1 mM NO, 4 mM ascorbate, 100 μM DETAPAC and 4 mM UQ-0 in 50 mM phosphate buffer (pH 7.4) using a NO-specific electrochemical probe (ISO-NOP). After 30 min of mixing all reactants, a sample of the gaseous phase was withdrawn from the reaction vessel and injected in the chromatograph. Quantification of N_2O

production was performed at 37°C by determining the total amount of N_2O in the gas and aqueous phases using the reported Henry's constant for N_2O at this temperature[30] to obtain the amount at the liquid phase.

RESULTS AND DISCUSSION

Nitric Oxide Reduction Kinetics

Changes in NO levels as a function of time in the reaction mixture containing DEA/NO and ascorbate in deareated phosphate buffer in the absence and presence of various quinones was monitored using the NO specific electrode and results are presented in Fig. 2. Initial rates were measured from the initial slope of the [NO] traces where only a small percent of all reactants have reacted and thus their concentrations are essentially constant. NO and ascorbate concentrations were selected so that this was possible, i.e. as shown in Fig. 2, initial straight line portions of these curves permitted the discrimination between initial rates between the different runs. In the absence of quinone, a small change in the NO levels as a function of time was noted in the reaction mixture containing DEA/NO and ascorbate (Fig. 2, curve (a)). However, when quinones are included in this reaction, at 10 μM concentration, a relatively rapid decrease of NO was noted (Fig. 2, curves (b)–(e)). The increase in NO consumption followed the order UQ-0 > NQ > TMQ > MNQ. When UQ-0 was tested at 10 and 80 μM, a dose dependent enhancement in NO consumption as a function of time was noted (Fig. 2, curves (e) and (f)).

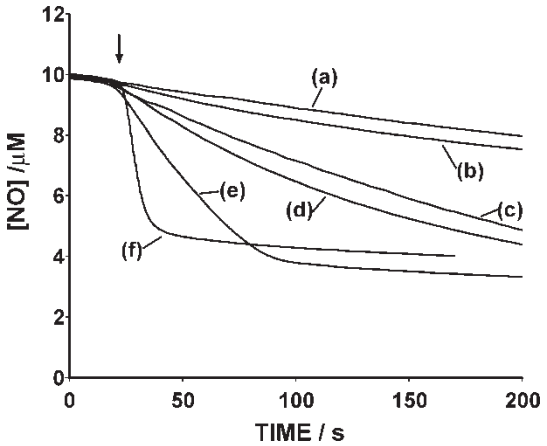

FIGURE 2 Nitric oxide consumption. Nitric oxide concentration traces occurring in N_2-saturated samples containing 50 mM phosphate buffer (pH 7.4), 100 μM DETAPAC, 10 μM ascorbate and (a) no quinone, (b) 10 μM MNQ, (c) 10 μM TMQ, (d) 10 μM NQ, (e) 10 μM UQ-0, (f) 80 μM UQ-0. The arrow indicates the instant where ascorbate was injected to the sample. Runs on this Figure were made the same day and representative curves are shown. However, data reproducibility is shown on Fig. 3 and on Table I.

Plots of the initial rates of NO consumption after subtracting the NO consumption rate in the absence of quinone, $R_{NO,0,}$ as a function of quinone concentration, for a constant ascorbate concentration, and as a function of ascorbate concentration, for a constant quinone concentration, are linear (Fig. 3). Thus, orders with respect to both quinone and ascorbate are one, respectively, and Eq. (3) is obeyed. If these rates are plotted against the product [Q] [AH^-], a straight line is produced for each

$$R_{NO,0} = k_{obs}[Q][AH^-] \quad (3)$$

of the quinones under study (Fig. 3(c)), from which k_{obs} can be obtained (Table I). This value of k_{obs} increases with an increase in the quinone E_7^1. value (Table I, Fig. 3(d)) and are within the range of the "effective" rate constants of quinone-catalyzed ascorbate oxidation reactions (i.e. from 10^0 to $10^3\,M^{-1}\,s^{-1}$).[4]

The hydroquinone derivatives of these quinones, i.e. the two-electron reduced product could also be involved in this electron transfer process. For example, the hydroquinone of UQ-0 has been reported to reduce NO with a rate constant of $4.9 \times 10^5\,M^{-1}\,s^{-1}$.[31] However, ascorbate is a one-electron reducing agent. Thus, the hydroquinone species, QH_2, can only be produced through the semiquinone disproportionation reaction (reaction (4)) or through the one-electron reduction of the

$$2Q^{\cdot -} + 2H^+ \rightarrow Q + QH_2 \quad (4)$$

semiquinone (reaction (5)). The rate constant of semiquinone disproportionation is in the order of

$$Q^{\cdot -} + AH^- + H^+ \rightarrow QH_2 + A^{\cdot -} \quad (5)$$

$10^8\,M^{-1}\,s^{-1}$.[32] In addition, the rate constant of reaction (5) has been estimated in the order of $10^5\,M^{-1}\,s^{-1}$.[5] Thus, assuming a steady state concentration for the semiquinone of 1 μM (estimated for an initial quinone concentration in the order of 10 μM) and a 10^{-5} M concentration of ascorbate, one can calculate rates of QH_2 formation in the order of 10^{-6} to 10^{-4} M/s. Since the rates measured in the present work are in the order of 10^{-7} M/s, the possibility exists that hydroquinones are also involved in the NO reduction process.

Evidence of Nitroxyl Formation

Formation of N_2O is unambiguous evidence for HNO production or NO reduction and has been used as the sole evidence of HNO in several works.[33–38] Head space sampling of the nitrogen-saturated reaction mixture containing DEA/NO, ascorbate and in the presence or absence of quinone demonstrated the formation of N_2O as determined by GC (Table I). The amount of N_2O detected after 30 min of

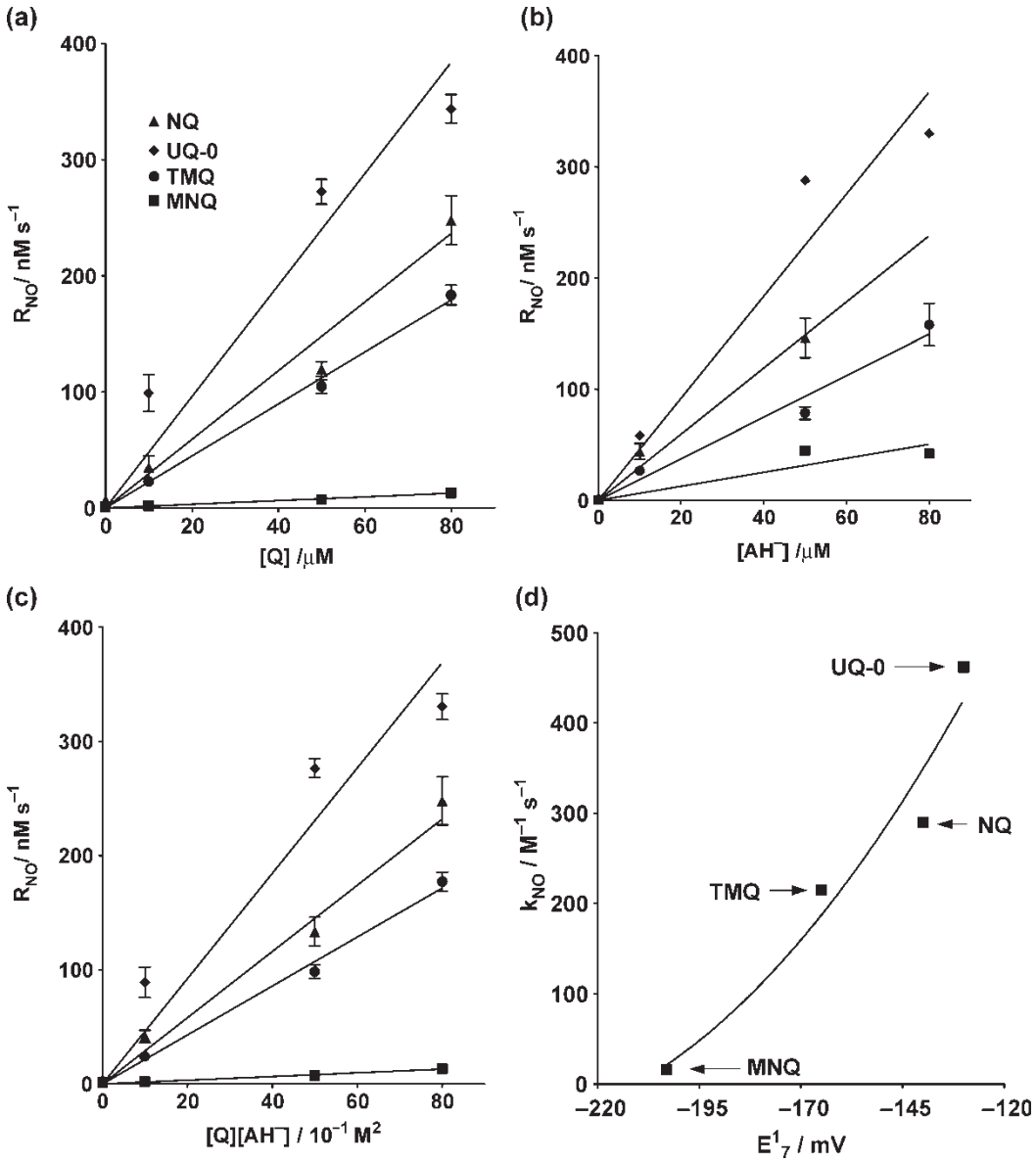

FIGURE 3 Dependence of the initial rate of the anaerobic NO consumption, at 37°C and pH 7.4, on quinone and ascorbate concentrations. Graphs correspond to solutions containing (a) 10 μM ascorbate and several initial concentrations of quinone, (b) 10 μM quinone and several initial concentrations of ascorbate. Graph (c) correspond to the product of quinone and ascorbate concentrations used in (a) and (b). Graph (d) shows the dependence of the initial NO consumption rate constant, determined from graphs shown in (c), on the quinone redox potential.

initiating the reaction was also found to increase with quinone redox potential. Thus, this is evidence that N_2O is being produced due to either the reaction of HNO with NO (reaction (6)) or HNO dimerization followed by dehydration (reaction (7)).[39,40]

$$^1HNO + 2NO \rightarrow N_2O + HNO_2 \quad (6)$$

$$^1HNO + {}^1HNO \rightarrow N_2O + H_2O \quad (7)$$

TABLE I Values of k_{obs}, E^1_7, and relative amount of N_2O produced, corresponding to quinones under study here

Quinone	E^1_7 (mV)	k_{obs} ($M^{-1}s^{-1}$)	Relative N_2O amount*
UQ-0	−130†	462 ± 18	100 ± 9
NQ	−140‡	290 ± 14	81 ± 6
TMQ	−165b‡	215 ± 6	68 ± 4
MNQ	−203b‡	16 ± 2	48 ± 4
None	–	–	38 ± 3

*Relative N_2O GC peak area determined as described in the "Materials and methods" section. †As estimated in Ref. [49]. ‡From Ref. [50].

The measured mol ratio of N_2O produced to that of NO consumed, corresponding to samples containing UQ-0, was found to be 0.44 ± 0.07. Dimerization of HNO to produce N_2O is a relatively slow process, and it is thus likely that N_2O is formed mostly from reaction (6). Indeed, the expected stoichiometry for this process (mole N_2O appearing/mole NO disappearing) is predicted to be 0.33, relatively close to the observed value of 0.44. The disappearance of NO is not due to non-redox reactions of NO with

the radical quinone species or with the ascorbyl radical since, if this were the case, a mol ratio of N_2O produced to NO consumed of 0.44 would not be obtained. Nitrous oxide is evidence for NO reduction. A reaction of NO with the semiquinone which does not involve electron transfer from the semiquinone (or the hydroquinone) to NO would have produced a smaller value than 0.33 (the ratio predicted if reaction (6) is occurring) for the N_2O produced/NO consumed mol ratio and that is not the case. The same reasoning applies to a possible reaction of the ascorbyl radical with NO. Ascorbyl oxidation by NO should not occur since ascorbate oxidation is more probable due to a much larger ascorbate concentration and to the positive reduction potential of ascorbyl (versus NHE).

Although several works suggest direct reduction of NO by biologically relevant molecules,[41–43] it has been recently argued that NO can not be reduced directly by common biological reducing agents since the NO redox potential is too negative. From the p*K*a value of 11.6 for ^{1}HNO ($^1\text{HNO} \rightarrow {}^3\text{NO}^- + \text{H}^+$), a potential of about -0.5 V (1 M versus NHE) has been estimated for the reduction of NO to $^3\text{NO}^-$ and subsequent protonation to HNO at pH 7.2.[19] Thus, direct reduction of NO by ascorbate ($E_7^1 = +0.282$ V (1 M versus NHE) for the ascorbyl radical[44]) should not be probable. However, it is recognized that a given redox reaction with a negative redox potential will occur if some of its products is used in other reactions.[45] Formation of N_2O and its release into the gaseous phase could be such reaction. The value of 11.6 for the p*K*a of $^3\text{NO}^-$ is for the formation of the singlet HNO; this protonation is a spin-forbidden process, and it is highly likely that, even with such a high p*K*a, the $^3\text{NO}^-$ exhibits a long lifetime which has been estimated in the order of miliseconds[20] (i.e. is protonated slowly). Thus, $^3\text{NO}^-$ could react with other species, in particular, with 2 NO molecules to generate N_2O and HNO_2 (reaction (6)). This would mean that the protonation of $^3\text{NO}^-$ should not be occurring at conditions where NO is in excess. Even though quinones also have negative redox potentials, these are just catalyzing the NO reduction reaction, i.e. if quinone reduction and semiquinone oxidation are included in this thermochemical cycle their reduction and oxidation potentials are, of course, cancelled out. In a similar manner, quinones catalyze oxygen reduction by ascorbate.[4,5]

The reduction potential of the NO, $\text{H}^+/{}^1\text{HNO}$ couple is also comparable (-0.55 V at pH 7)[20] with the NO/$^3\text{NO}^-$ couple at pH 7 and could be postulated as another path for the NO reduction step. This is due in large part to the very strong basicity of $^1\text{NO}^-$ (p*K*a = 23).[20] A possibility for NO reduction, without these large energy barriers, could involve an inner sphere reduction of NO whereby the semiquinone forms a complex with NO followed by electron transfer from the semiquinone to NO. Furthermore, at relatively high concentrations of NO a dimer is formed which is more prone for reduction (+0.33 V versus NHE[19]). In view of the fact that, as stated above, NO production is enhanced under hypoxia and under certain pathological conditions,[46–48] the observations obtained in this work should even be more relevant under such conditions.

In summary, the quinones under study here enhance the ascorbate reduction of NO. The observed rate constant for this reaction increases with an increase in the quinone E_7^1 value. Evidence has been obtained to sustain the reduction of NO to nitroxyl.

Acknowledgements

The authors express appreciation for grants No. S06-GM08216 and R15 CA82142 from NIH for financial support of this work and to Drs Murali Krishna from NCI (USA) and Amram Samuni from Hebrew University Medical School for helpful discussions.

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