Oxidation of ubiquinol by peroxynitrite: implications for protection of mitochondria against nitrosative damage

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A major pathway of nitric oxide utilization in mitochondria is its conversion to peroxynitrite, a species involved in biomolecule damage via oxidation, hydroxylation and nitration reactions. In the present study the potential role of mitochondrial ubiquinol in protecting against peroxynitrite-mediated damage is examined and the requirements of the mitochondrial redox status that support this function of ubiquinol are established. (1) Absorption and EPR spectroscopy studies revealed that the reactions involved in the ubiquinol/peroxynitrite interaction were first-order in peroxynitrite and zero-order in ubiquinol, in agreement with the rate-limiting formation of a reactive intermediate formed during the isomerization of peroxynitrite to nitrate. Ubiquinol oxidation occurred in one-electron transfer steps as indicated by the formation of ubisemiquinone. (2) Peroxynitrite promoted, in a concentration-dependent manner, the formation of superoxide anion by mitochondrial membranes. (3) Ubiquinol protected against peroxynitrite-mediated nitration of tyrosine residues in albumin and mitochondrial membranes, as suggested by experimental models, entailing either addition of ubiquinol or expansion of the mitochondrial ubiquinol pool caused by selective inhibitors of complexes III and IV. (4) Increase in membrane-bound ubiquinol partially prevented the loss of mitochondrial respiratory function induced by peroxynitrite. These findings are analysed in terms of the redox transitions of ubiquinone linked to both nitrogen-centred radical scavenging and oxygen-centred radical production. It may be concluded that the reaction of mitochondrial ubiquinol with peroxynitrite is part of a complex regulatory mechanism with implications for mitochondrial function and integrity.

Key words: cytochrome oxidase, nitric oxide, superoxide anion, superoxide dismutase.

INTRODUCTION

The effects of $^{\bullet}NO$ on the regulation of mitochondrial O_2 uptake are of importance for pathological conditions associated with increased levels of $^{\bullet}NO$ [1]. The intramitochondrial steady-state concentration of $^{\bullet}NO$ ranges from 20–500 nM depending on, on the one hand, the sources of $^{\bullet}NO$ related to the activities of nitric oxide synthase (NOS) isoforms [mitochondrial (mtNOS), endothelial (eNOS), neuronal (nNOS) and inducible (iNOS)] and, on the other, the mitochondrial pathways for $^{\bullet}NO$ utilization (Scheme 1). These involve reductive and oxidative decay mechanisms [2]. The former encompasses a general reduction of $^{\bullet}NO$ to the nitroxyl anion (NO^-) and occurs at the expense of electron donation, mainly from ubiquinol [3] and, to a lesser extent, from cytochrome *c* [4] and cytochrome oxidase [5]. The latter, the oxidative decay mechanisms, involve the reaction of $^{\bullet}NO$ with O_2^- to yield $ONOO^-$, a reaction that takes place at diffusion controlled rates ($k = 1.9 \times 10^{10}\ M^{-1} \cdot s^{-1}$) [6].

The reductive and oxidative decay pathways of $^{\bullet}NO$ in mitochondria (under conditions entailing an expanded ubiquinol pool) are linked to the redox transitions of ubiquinone [2]; reduction of $^{\bullet}NO$ by ubiquinol generates ubisemiquinone (eqn. 1), which decays by autoxidation to generate O_2^- (eqn. 2); the rapid reaction of the latter with $^{\bullet}NO$ leads to $ONOO^-$ generation (eqn. 3):

$$^{\bullet}NO + UQH^- \rightarrow NO^- + UQ^{\bullet -} + H^+ \quad (1)$$

$$UQ^{\bullet -} + O_2 \rightarrow UQ + O_2^{\bullet -} \quad (2)$$

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow ONOO^- \quad (3)$$

where UQH^- is ubiquinol, $UQ^{\bullet -}$ is ubisemiquinone, and UQ is ubiquinone.

In agreement with these notions, it was observed that in respiring mitochondria $^{\bullet}NO$ decays largely through $ONOO^-$ formation [2], and that diaphragm mitochondria undergo loss of integrity and function associated with protein nitration after exposure to increased endogenous $^{\bullet}NO$ and $ONOO^-$ production [1]. Furthermore, the implications for removal of $^{\bullet}NO$ (eqn. 3) are twofold: on the one hand, it releases cytochrome oxidase inhibition and, on the other hand, it leads to $ONOO^-$ formation.

In spite of its short half-life, peroxynitrite reacts with a wide range of biomolecules, such as proteins [7], nucleotides [8], lipids [9] and antioxidant molecules [10,11]. 3-Nitrotyrosine, a fingerprint of peroxynitrite reactivity towards tyrosine residues in proteins, has been found in physiological and pathological conditions [12,13]. Considering that mitochondrial membranes are permeable to $^{\bullet}NO$ and impermeable to $O_2^{\bullet -}$ and that NO in mitochondria decays largely via $ONOO^-$ formation, it seems pertinent to define physiologically-relevant mitochondrial mechanisms of protection against $ONOO^-$-mediated damage. In this context, the role of ubiquinol as an antioxidant gains significance

Abbreviations used: NOS, nitric oxide synthase; DTPA, diethylenetriaminepenta-acetic acid; UQ_0, 2,3-dimethoxy-6-methyl-1,4-benzoquinone; UQ_2, decylubiquinone; UQ_{10}, ubiquinone-50.

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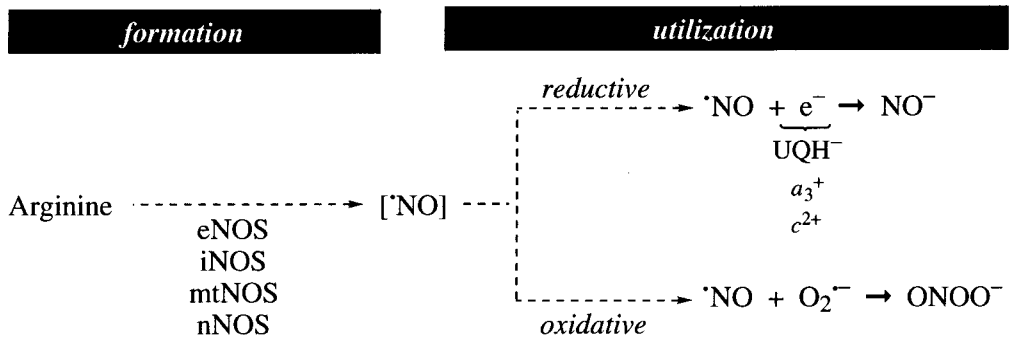


Scheme 1 Reductive and oxidative decay pathways of nitric oxide in mitochondria

UQH^-, ubiquinol; c^{2+}, ferrocytochrome *c*; a_3^+, reduced cytochrome oxidase. Explanations are given in the text. eNOS, endothelial NOS; iNOS, inducible NOS; mtNOS, mitochondrial NOS; nNOS, neuronal NOS.

in view of its ubiquitous distribution and its effective recovery by electrons channeled through mitochondrial complexes I and II. Hence, the goals of this study were to characterize the potential reaction of ubiquinol with $ONOO^-$, examine the mitochondrial redox conditions favouring such a reaction, and to establish a role for ubiquinol in protection against $ONOO^-$-mediated nitration processes.

MATERIALS AND METHODS

Chemicals and biochemicals

2,3-Dimethoxy-6-methyl-1,4-benzoquinone (UQ_0), decylubiquinone, and ubiquinone-50 (Q_{10}), diethylenetriaminepenta-acetic acid (DTPA), H_2O_2, $NaNO_2$, KBH_4, NaCN, myxothiazol, antimycin A, fatty acid-free BSA and superoxide dismutase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acrylamide solutions, nitrocellulose membranes and goat anti-rabbit IgG were from Bio-Rad (Hercules, CA, U.S.A.). Specific anti-nitrotyrosine polyclonal antibody was a gift from Dr. Alvaro Estevez (University of Alabama at Birmingham, AL, U.S.A.). All other reagents were of analytical grade.

Peroxynitrite was synthesized in a quenched flow reactor from 0.7 M $NaNO_2$ and 0.7 M H_2O_2, and stabilized with 1.2 M NaOH, as described previously [14]. H_2O_2 was removed by adding granular MnO_2. The solution was frozen at -70 °C. Peroxynitrite, concentrated in the yellow top layer was collected and its concentration was determined spectrophotometrically ($\epsilon_{302} = 1.67\ mM^{-1}\cdot cm^{-1}$) [14].

Chemical reduction of either UQ_0 or UQ_2 was obtained upon addition of 40μl of KBH_4 (0.5 M dissolved in 0.1 M NaOH) to 1 ml of 20 mM quinone dissolved in either water (UQ_0) or ethanol (UQ_2); excess KBH_4 was removed by treatment with HCl [15]. Ubiquinol solutions were purged with argon for 5 min in a sealed flask.

Isolation of rat liver mitochondria, preparation of submitochondrial particles, and determination of ubiquinone content

Rat liver mitochondria were isolated in 0.23 M mannitol/70 mM sucrose/1 mM EDTA/10 mM Tris/HCl, pH 7.3, as described previously [16]. Submitochondrial particles were prepared from frozen and thawed liver mitochondria (20 mg protein/ml) placed in a small beaker in an ice bath, and disrupted by sonication for three 10 s periods with 30 s intervals at an output of 40 W using a Model W-225R sonifier (Heat Systems/Ultrasonics, Chicago, IL, U.S.A.) [17]. The submitochondrial particles were washed three times and resuspended in the above buffer at a concentration of 10 mg protein/ml. All operations were performed at 0–4 °C.

Increase in the steady-state level of endogenous ubiquinol was achieved by supplementing submitochondrial particles with succinate in the presence of complex III/IV inhibitors: antimycin A was used as a complex III inhibitor at the site of cytochromes $b_{562-566}$, myxothiazol was used as a complex III inhibitor at the Rieske Fe-S protein [18], and cyanide as a cytochrome oxidase inhibitor. Increase in the membrane ubiquinone pool was achieved by incubating submitochondrial particles (2.5 mg/ml) with various amounts of UQ_2 for 30 min in 100 mM phosphate buffer, pH 7.4, at 30 °C; reduction of added UQ_2 was accomplished upon supplementation of the membranes with 6 mM succinate and 2.4 μM myxothiazol.

Rats were injected intramuscularly with a single dose of UQ_{10} (20 mg/kg) suspended in aqueous soybean lecithin (1:1, v/v). Animals were killed 16 h after injection. Ubiquinone content in mitochondria (isolated as described above) was determined after extraction with cyclohexane/ethanol (5:2, v/v) (6 mg of mitochondrial protein/ml and 7 ml of cyclohexane/ethanol) [19]. Total ubiquinone content was calculated using the molecular absorption coefficient for the difference in absorption of the oxidized and reduced (obtained after addition of 0.2 mg KBH_4) forms of ubiquinone ($\epsilon_{ox} - \epsilon_{red}$) at A_{275} ($\Delta\epsilon = 12.25\ mM^{-1}\cdot cm^{-1}$) [20].

Absorption spectroscopy

Ubiquinol spectral changes were followed using a Hitachi U-3000 spectrophometer (Hitachi Co. Ltd., Tokyo, Japan) in the 220–340 nm range ($\epsilon_{268} = 13.8\ mM^{-1}\cdot cm^{-1}$) [21]. The effect of UQ_0H_2 on the rate of peroxynitrite decomposition was assessed in a stopped-flow spectrophotometer (Applied Photophysics SF.17MV) with dead time of < 2 ms. The temperature was maintained at 37.0 ± 0.1 °C. A wavelength of 304 nm was used to minimize interference of UQ_0H_2 or UQ_0 absorbance. Changes in pH because of the addition of acidic solutions of UQ_0H_2 were counteracted by adding NaOH, and the pH was measured after the reactions. To monitor the effect of decomposition products of KBH_4 present in UQ_0H_2 solutions, peroxynitrite was mixed with control solutions prepared in the absence of UQ_0H_2. To prevent the reaction of UQ_0H_2 or semiquinone radical with oxygen, the solutions were degassed with argon for 15 min before

mixing in the stopped-flow spectrophotometer. Apparent rate constants were determined by non-linear least-squares fitting of stopped-flow data to a single exponential function using the software provided with the instrument. The values reported are the average of at least seven determinations. $O_2^{\bullet-}$ production by rat liver submitochondrial particles was measured by superoxide-dismutase-sensitive cytochrome *c* reduction ($\epsilon_{550} = 21.1\ mM^{-1} \cdot cm^{-1}$, as described previously [22].

EPR spectroscopy

EPR spectra were recorded on a Bruker ECS 106 spectrometer (Bruker Analytik GmbH, Rheinstetten, Germany) equipped with a TM 8810 microwave cavity. Measurements were carried out at room temperature at a microwave frequency of 9.80 GHz and 100 kHz field modulation. Continuous-flow EPR measurements were performed with argon-purged solutions of $ONOO^-$ (400 μM in 10 mM NaOH) and UQ_0H_2 (400 μm in 200 mM phosphate buffer, pH 7.4) mixed before the cavity at a flow rate of 7 ml/min.

Immunoblotting and nitrotyrosine detection

BSA or submitochondrial particles (20 μg or 25 μg/lane respectively) were separated by electrophoresis on precast SDS 7.5% polyacrylamide gel and transferred on to a PVDF membrane. The membranes were incubated with a rabbit anti-3-nitrotyrosine polyclonal antibody (1:2000) and blotted with a goat anti-rabbit IgG (1:3000) conjugated to alkaline phosphatase (Bio-Rad) followed by detection of immunoreactive proteins by a chemiluminescence method. BSA, exposed or not to 1 mM $ONOO^-$, was used as control.

RESULTS

Ubiquinol oxidation by peroxynitrite

Absorption spectral analysis of an anaerobic solution of UQ_0H_2 in the UV (220–340 nm) region revealed a maximum at 294 nm (Figure 1A). Addition of $ONOO^-$ (45 μM pulses) resulted in a progressive increase in absorption at 268 nm with isosbestic points at 232 and 289 nm, spectral changes ascribed to ubiquinone formation. The amount of ubiquinol oxidized was linearly related to the amount of $ONOO^-$ added, up to a ratio of $[ONOO^-]/[UQ_0H_2] \sim 4$; beyond this value, the amount of peroxynitrite required to obtain total ubiquinol oxidation did not follow a linear relationship. The amount of ubiquinol oxidized represented 22% of $ONOO^-$ added for the first three pulses (Figure 1B).

As observed in the time-dependent spectra shown in Figure 2(A), $ONOO^-$ decomposition in the presence of ubiquinol was accompanied by an increase in absorbance in the 400–450 nm region, due to quinol oxidation. However, the rate of $ONOO^-$ decomposition ($0.86 \pm 0.02\ s^{-1}$; Figure 2B) was not increased in the presence of ubiquinol ($0.82 \pm 0.03\ s^{-1}$; Figure 2C).

The kinetic pattern in Figure 1 and the time courses in Figure 2 indicate that the reactions involved in the $ONOO^-/UQ_0H_2$ interaction are first-order in $ONOO^-$ and zero-order in UQ_0H_2. This is in agreement with the rate-limiting formation of a reactive intermediate formed during the isomerization of $ONOO^-$ to NO_3^- (eqns. 4–6):

$$ONOO^- + H^+ \rightarrow ONOOH \quad (4)$$

$$ONOOH \rightarrow [HO^\bullet + {}^\bullet NO_2] \quad (5)$$

$$[HO^\bullet + {}^\bullet NO_2] \rightarrow H^+ + NO_3^- \quad (6)$$

This intermediate, which is capable of nitration, hydroxylation and oxidation, may decompose via homolytic O–O bond cleavage

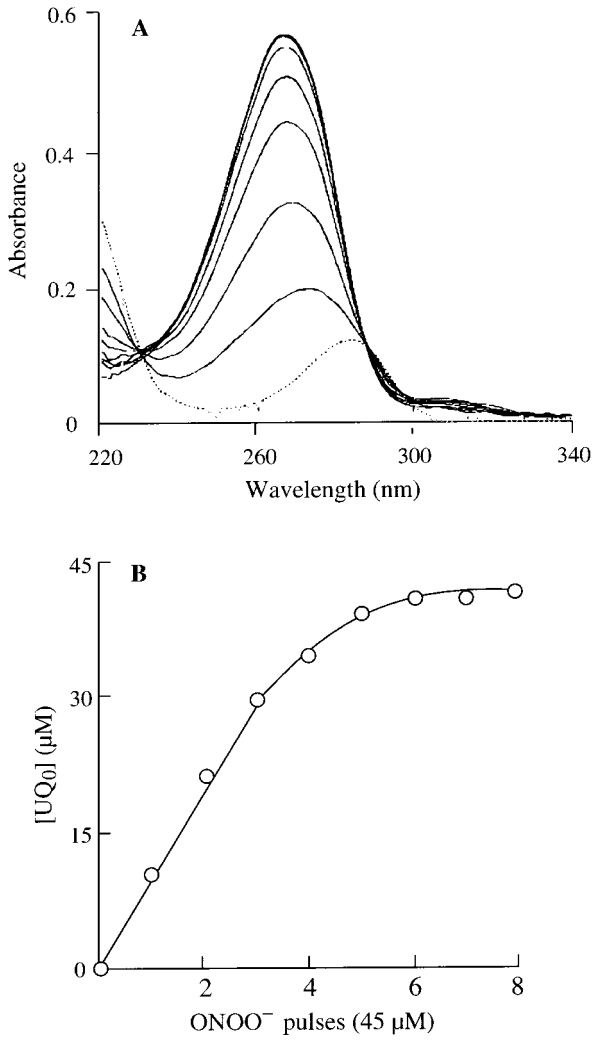


Figure 1 Oxidation of ubiquinol by peroxynitrite

(**A**) UV absorption spectrum. Conditions used: anaerobic solution of 45 μM UQ_0H_2 in 100 mM sodium phosphate buffer, pH 7.4, containing 1 mM DTPA was supplemented with various amounts of peroxynitrite (45 μM pulses). (**B**) Dependence of ubiquinone formation upon peroxynitrite concentration. Data were obtained from the A_{268} shown in (**A**).

to yield $HO^\bullet$ and $NO_2^\bullet$ (eqn. 5); the recombination of these two radicals can result in NO_3^- formation (eqn. 6) [23–28]. The experiments in Figures 1 and 2 were carried out in argon-purged solutions, thereby ruling out a role for reactive intermediates originating from the reaction of $ONOO^-$ and CO_2.

Formation of ubisemiquinone during the oxidation of ubiquinol by peroxynitrite

The oxidation of ubiquinol by the above mentioned reactive intermediate is expected to proceed in one-electron transfer steps; this notion is strengthened by the detection by continuous flow EPR of a signal [hyperfine splitting constants: a^H (3H) = 2.14 G, a^H (1H) = 1.72 G; line intensity ratio: 13.5:5:3:1] ascribed to ubisemiquinone formed during the interaction of UQ_0H_2 and $ONOO^-$ in anaerobic conditions (Figure 3A) (eqn. 7):

$$HO^\bullet + {}^\bullet NO_2 + UQH^- \rightarrow HO^- + NO_2^\bullet + UQ^{\bullet-} + H^+ \quad (7)$$

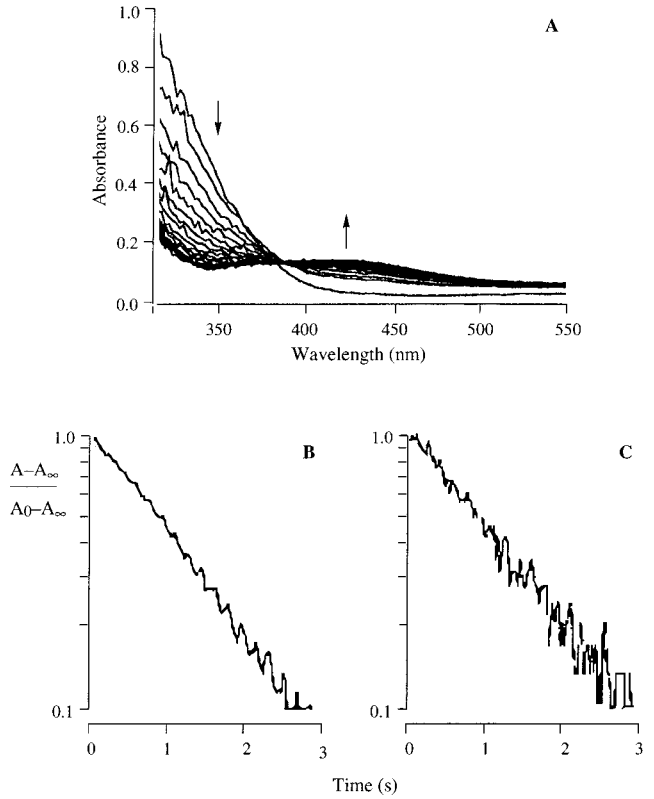


Figure 2 Time-course traces of $ONOO^-$ decomposition

(**A**) Time-dependent spectra obtained for $ONOO^-$ decomposition in the presence of UQ_0H_2. $ONOO^-$ (1 mM) was mixed with 0.1 M phosphate buffer, pH 7.37, containing 0.1 mM DTPA in the presence of 1 mM UQ_0H_2. The time span between consecutive spectra was 346 ms. The solutions were degassed with argon for 15 min before mixing. (**B**, **C**). Time courses of $ONOO^-$ spontaneous decomposition. $ONOO^-$ (0.1 mM) was mixed with 0.1 mM phosphate buffer (pH 7.37) (**B**) containing 0.1 mM DTPA at 37 °C, in the absence (**B**) or presence (**C**) of 5 mM UQ_0H_2. The solutions were degassed with argon for 15 min before mixing.

The EPR signal was short lived in experiments where continuous flow was not used, probably due to the decay of the ubisemiquinone by disproportionation (eqn. 8):

$$UQ^{\bullet -} + UQH^{\bullet} \rightarrow UQ + UQH^- \quad (8)$$

where UQH^- is ubiquinol, $UQ^{\bullet -}$ is ubisemiquinone, and UQ is ubiquinone. The rate of eqn. (7) is expected to be fast, assuming $HO^{\bullet}$ or $NO_2^{\bullet}$-like chemistry of the intermediate. For example, the second-order rate constant for the reaction of benzohydroquinone with $HO^{\bullet}$ and $NO_2^{\bullet}$ is $> 10^9\ M^{-1} \cdot s^{-1}$ and $\sim 5 \times 10^8\ M^{-1} \cdot s^{-1}$ respectively [29]. The rate of eqn. (8) depends on the relative concentrations of the anionic ($UQ^{\bullet -}$) and protonated ($UQH^{\bullet}$) forms of the semiquinone and, at pH 7.4, it occurs with a second-order rate constant of $\sim 8 \times 10^4\ M^{-1} \cdot s^{-1}$ [30].

Peroxynitrite-dependent ubiquinol oxidation and superoxide anion formation

The formation of ubisemiquinone during the oxidation of ubiquinol by peroxynitrite (Figure 2; eqn. 7) suggests an additional source of $O_2^{\bullet -}$ in mitochondrial membranes upon autoxidation of the ubisemiquinone (eqn. 9):

$$UQ^{\bullet -} + O_2 \rightarrow UQ + O_2^{\bullet -} \quad (9)$$

Accordingly, under conditions entailing an enhanced level of endogenous ubiquinol in mitochondrial membranes (submitochondrial particles supplemented with succinate in the presence of myxothiazol), $ONOO^-$ elicited $O_2^{\bullet -}$ formation (Figure 4). The rate of $O_2^{\bullet -}$ production and total $O_2^{\bullet -}$ production were linearly related to protein (submitochondrial particles) concentration. Under these conditions, the $O_2^{\bullet -}$ production rate increased with increasing concentrations of $ONOO^-$ (Figure 4 inset).

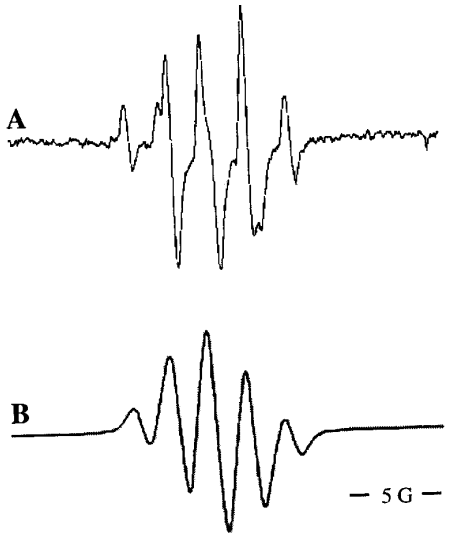


Figure 3 $ONOO^-$-mediated ubisemiquinone formation

(**A**) Continuous-flow EPR spectrum of $UQ_0^{\bullet -}$. Solutions of 400 μM UQ_0H_2 and $ONOO^-$ (in 10 mM NaOH) in argon-purged 0.2 M phosphate buffer, pH 7.4, were mixed at a flow rate of 7 ml/min. (**B**) Simulated spectrum of (**A**). Instrument settings: modulation amplitude, 0.963 G; modulation frequency, 50.0 kHz; microwave power, 31.7 mW; sweep width, 30 G; sweep rate, 18 G/min; conversion time, 163.64 ms.

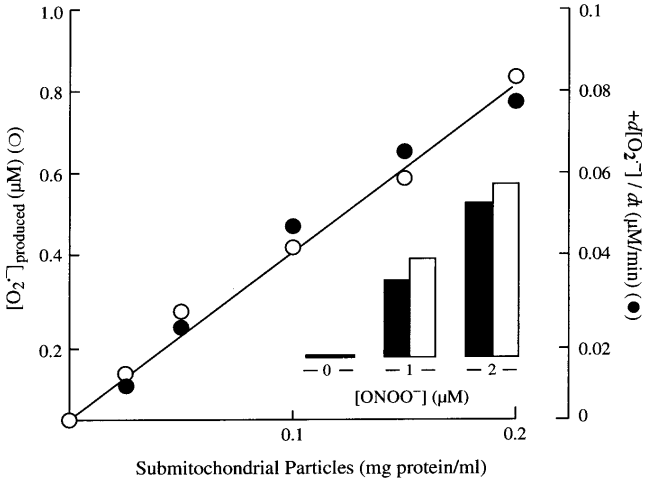


Figure 4 $ONOO^-$-dependent O_2^- generation by submitochondrial particles

Assay conditions: submitochondrial particles (0.05–0.2 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, were supplemented with 6 mM succinate and 2.4 μM myxothiazol. The reaction was initiated by the addition of 1 μM $ONOO^-$. Inset: effect of varying concentrations of $ONOO^-$ on $O_2^{\bullet -}$ generation. Assay conditions: submitochondrial particles (0.1 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, were supplemented with 6 mM succinate and 2.4 μM myxothiazol in the presence of various amounts of $ONOO^-$. White bars, $[O_2^{\bullet -}]_{produced}$ (μM); black bars, $d[O_2^{\bullet -}]/dt$ (μM/min).

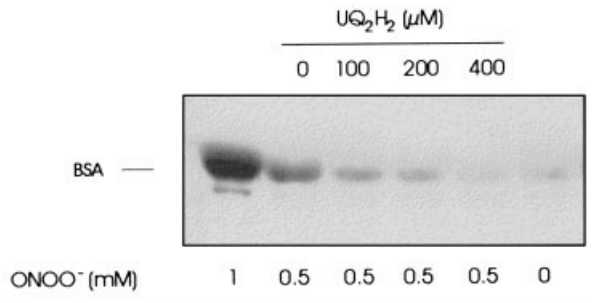


Figure 5 Effect of ubiquinol on peroxynitrite-mediated nitration of BSA

Assay conditions: BSA (2 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, was supplemented with 0.5 mM $ONOO^-$ and various amounts of UQ_2H_2 (0–400 μM). The assay was performed as described in the Materials and methods section.

Effect of ubiquinol on peroxynitrite-mediated protein nitration

The reaction of ubiquinol with a reactive intermediate (eqn. 7) suggests a role for this electron donor in pathways inherent in the chemical reactivity of this intermediate, i.e. nitration, hydroxylation and oxidation. This notion was examined with experimental models involving tyrosine nitration, a fingerprint of $ONOO^-$ action, in BSA and mitochondrial proteins by Western blotting.

Exposure of BSA to $ONOO^-$ resulted in nitrotyrosine immunoreactivity (Figure 5); the intensity of this band was decreased with increasing amounts of ubiquinol (in the 0–400 μM range), thereby suggesting competition between ubiquinol and the tyrosine residue in the protein for the reactive intermediate involved in nitration. The reaction of $ONOO^-$ with BSA proceeded with a second-order rate constant of $7.5 \pm 0.8 \times 10^3\ M^{-1} \cdot s^{-1}$ [24]; it may be inferred that, under the experimental conditions depicted in Figure 5 (30 μM BSA and 0.5 mM $ONOO^-$), approx. 18 % of $ONOO^-$ reacted directly with BSA, and most of it decayed with production of secondary oxidants.

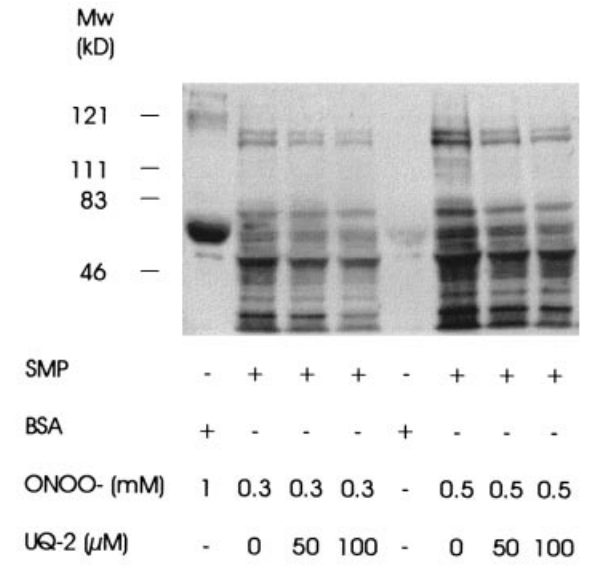


Figure 6 Effect of exogenous ubiquinone on $ONOO^-$-mediated nitration of submitochondrial particles

Assay conditions: submitochondrial particles (SMP) (2.5 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, in the presence of 6 mM succinate and 2.4 μM myxothiazol were supplemented with either 0.3 or 0.5 mM $ONOO^-$ and various amounts of UQ_2 (0–100 μM). Controls consisted of BSA (2 mg protein/ml) supplemented with 1 mM $ONOO^-$. Molecular-mass markers in kDa (MW, kD) are shown on the left.

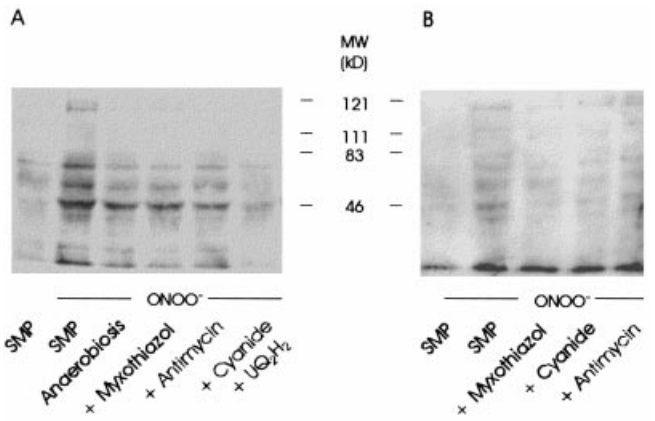


Figure 7 Effect of exogenous and endogenous ubiquinone redox status on $ONOO^-$-mediated nitration of submitochondrial particles

Assay conditions: (**A**) Submitochondrial particles (SMP) (2.5 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, were supplemented with 6 mM succinate, various electron-transfer inhibitors and 20 μM UQ_2. After 30 min incubation, the samples were exposed to a single pulse of 200 μM $ONOO^-$. (**B**) As in (**A**) but without the addition of UQ_2 and with 30 μM $ONOO^-$; data correspond to redox changes in endogenous ubiquinol. The individual concentration of inhibitors was: 2.4 μM myxothiazol, 2.4 μM antimycin A or 1 mM KCN. The positions of molecular-mass markers in kDa (MW, kD) are shown.

When incubated with $ONOO^-$, mitochondrial proteins exhibited reactivity for nitrotyrosine in numerous bands (Figure 6); the extent of tyrosine nitration was dependent on $ONOO^-$ concentration (only two concentrations, 0.3 and 0.5 mM, are shown in Figure 6). The effect of ubiquinol on nitration of tyrosine residues in mitochondrial proteins was assessed with experimental models involving manipulations of the capacity and redox status of the ubiquinol pool in membranes.

First, increase in the ubiquinol pool was achieved by supplementation of submitochondrial particles with varying amounts of UQ_2 in the presence of myxothiazol, and with succinate as electron donor, thereby resulting in reduction of UQ_2 and increase of the total ubiquinol pool. Under these conditions, a progressive decrease in nitrotyrosine immunoreactivity was observed with increasing amounts of ubiquinol (Figure 6).

Secondly, the redox status of the endogenous ubiquinol pool was increased by using inhibitors of the mitochondrial complexes III and IV (myxothiazol, antimycin A and cyanide), resulting in higher levels of endogenous ubiquinone in the reduced state. Under these conditions, a decrease in nitrotyrosine immunoreactivity, elicited by supplementation of mitochondrial membranes with $ONOO^-$, was observed (Figure 7).

Overall, data shown in Figures 5–7 strengthen the notion that the reaction of ubiquinol with the reactive intermediate formed in the decay of $ONOO^-$ to NO_3^- prevents nitration of tyrosine residues in a concentration-dependent manner. These effects of ubiquinol also suggest that the reaction of $ONOO^-$ with ubiquinol is a preferred decay pathway over the reaction of $ONOO^-$ with other reduced components of the respiratory chain, such as cytochrome *c* and cytochrome oxidase [4,31]. Accordingly, the role of the latter in protection against $ONOO^-$-dependent nitration may be less significant.

Table 1 Protective effects of parenteral administration of UQ_{10} on respiratory functions of rat liver mitochondria exposed to $ONOO^-$

Data are the means ± S.E.M. from 3–4 samples. Respiratory control is the ratio between O_2 uptake rates in the presence (state 3) and in the absence (state 4) of 0.2 mM ADP with 6 mM malate/glutamate as substrate. *Denotes $P < 0.05$ with respect to control samples (determined by Student's t test); †denotes $P < 0.05$ with respect to basal values by analysis of variance ('ANOVA') and Dunnett's test.

	Control	Injected with UQ_{10}
UQ_{10} content (μg/mg of protein)	1.29 ± 0.13	2.03 ± 0.17*
Respiratory control	8.1 ± 0.4	8.3 ± 0.4
+ 75 μM $ONOO^-$	6.1 ± 0.4	7.8 ± 0.4*
+ 150 μM $ONOO^-$	5.7 ± 0.4†	7.0 ± 0.3
+ 300 μM $ONOO^-$	4.3 ± 0.7†	4.5 ± 0.6†

Ubiquinone content and $ONOO^-$ concentration determine the oxidative damage of respiratory function in liver mitochondria

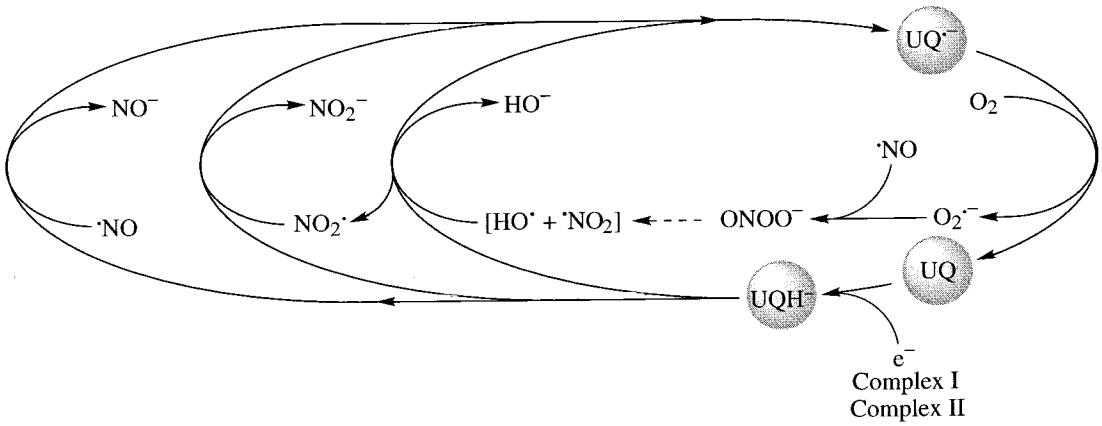
Mitochondrial content of UQ_{10} was increased ~ 57 % following the administration of the quinone to rats (Table 1), without changes in the respiratory control values. Supplementation of control mitochondria with 75, 150 or 300 μM $ONOO^-$ caused decreases in respiratory control values of ~ 25, 30, and 47 % respectively. The respiratory control values of liver mitochondria from rats injected with UQ_{10} were decreased by these $ONOO^-$ concentrations ~ 6, 15 and 45 % respectively (Table 1). It may be surmised that mitochondria with a higher content of UQ_{10} were endowed with adequate protection against damage caused by low $ONOO^-$ concentrations (e.g. 75 μM), whereas increased UQ_{10} content was apparently ineffective in protecting against functional damage caused by high $ONOO^-$ concentrations (e.g. 300 μM).

DISCUSSION

This study shows that (1) ubiquinol is oxidized by reactive intermediates formed in the decay of $ONOO^-$ to NO_3^- (the reaction being zero order in peroxynitrite; Figure 2) in one-electron transfer steps, as suggested by the generation of ubisemiquinone (Figure 3). (2) $ONOO^-$ promotes the formation of $O_2^{\bullet -}$ by mitochondrial membranes (Figure 4), probably via a reaction entailing ubiquinol oxidation to ubisemiquinone (eqn. 7) followed by autoxidation of the latter (eqn. 9). (3) Ubiquinol protects against $ONOO^-$-mediated nitration of tyrosine residues in BSA (Figure 5) and mitochondrial membranes (Figures 6 and 7), as surmised from experimental designs involving the addition of ubiquinol or an expanded ubiquinol pool in mitochondria caused by the action of selective complex III/IV inhibitors. (4) Increasing membrane-bound ubiquinol upon administration of UQ_{10} to rats partially prevents the $ONOO^-$-induced loss of mitochondrial respiratory function (Table 1).

These findings may be analysed in terms of the redox transitions of ubiquinone (ubiquinol → ubisemiquinone → ubiquinone) associated with, on the one hand, scavenging of free radicals (inherent in the ubiquinol → ubisemiquinone transition) downstream of peroxynitrous acid decomposition and, on the other hand, formation of $O_2^{\bullet -}$ (inherent in the ubisemiquinone → ubiquinone transition) (Scheme 2). It may be surmised that the occurrence of a reaction between mitochondrial ubiquinol and $ONOO^-$ involves regulatory and protective aspects: first, ubiquinol scavenges free radicals derived from peroxynitrous acid decomposition, thereby protecting mitochondrial proteins against nitration. Secondly, $ONOO^-$ elicits a concentration-dependent $O_2^{\bullet -}$ formation by mitochondrial membranes; this effect is ascribed to ubiquinol oxidation followed by ubisemiquinone autoxidation; further removal of $O_2^{\bullet -}$ by matrix Mn-superoxide dismutase yields H_2O_2 in turn reduced to H_2O by mitochondrial glutathione peroxidase. The sequences entailed in these radical decay pathways ($ONOO^- \rightarrow NO_2^{\bullet} \rightarrow NO_2^-$ and $O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow H_2O$) suggest a strong antioxidant effect exerted by membrane ubiquinol. Thirdly, decay of $O_2^{\bullet -}$ to either H_2O_2 (by a Mn-superoxide dismutase-catalysed reaction; $k = 2.3 \times 10^{10}\ M^{-1} \cdot s^{-1}$) or $ONOO^-$ (upon its fast reaction with $^{\bullet}NO$; $k = 1.9 \times 10^{10}\ M^{-1}\ s^{-1}$) is expected to be a function of the individual mitochondrial concentrations or steady-state levels of Mn-superoxide dismutase and $^{\bullet}NO$.

It is noteworthy that the reactions of ubiquinol with $^{\bullet}NO$ (eqn. 1) and peroxynitrite-derived radicals (eqn. 7) imply paradoxical effects: on the one hand, the higher utilization of $^{\bullet}NO$ involved



Scheme 2 Redox transitions of ubiquinol and the scavenging and formation of nitrogen- and oxygen-centred reactive species

The Scheme depicts: the scavenging of nitrogen-centred species coupled to the ubiquinol (UQH^-) → ubisemiquinone ($UQ^{\bullet -}$) redox transition; the formation of the superoxide anion coupled to the ubisemiquinone → ubiquinone (UQ) transition; and the transfer of electrons from complexes I and II to ubiquinone.

in its reductive decay to NO^- via ubiquinol favours the release of cytochrome oxidase inhibition and thereby restores mitochondrial O_2 uptake [32]. On the other hand, the ubiquinol-centred reactions are expected to amplify the formation of $ONOO^-$ (suggested by an enhanced ubisemiquinone autoxidation to yield $O_2^{\bullet -}$ and the fast reaction of the latter with $^\bullet NO$). The prevalence of these pathways is partly controlled by the intramitochondrial steady-state level of $^\bullet NO$ (in the range 0.05–0.5 μM); high steady-state concentrations of $^\bullet NO$ (as during inducible-NOS induction [1]) are expected to compete efficiently with Mn-superoxide dismutase, thus favouring $ONOO^-$ formation. Furthermore, the decay of the $ONOO^-$-derived radical upon reaction with ubiquinol is relevant for mitochondrial functions, because (a) ubiquinol is a unique component of the electron transfer chain, which is efficiently and continuously recycled via electrons from complexes I and II and (b) ubisemiquinone, formed as indicated in Figure 3, is a major source of oxyradicals in mitochondria.

Understanding of the protection exerted by ubiquinol against $ONOO^-$-mediated nitration of tyrosine residues requires consideration of the mechanistic aspects of this process. Formation of nitrotyrosine is a consequence of reactive species formed during the decay of $ONOO^-$ and it entails a sequence that involves H abstraction from tyrosine to yield a tyrosyl radical ($Tyr + HO^\bullet \rightarrow Tyr^\bullet + HO^-$) followed by $NO_2^\bullet$ addition to the latter ($Tyr^\bullet + NO_2^\bullet \rightarrow$ 3-nitrotyrosine; $k = 3 \times 10^{10}\ M^{-1} \cdot s^{-1}$) [33]. Hence, prevention of nitrotyrosine formation by ubiquinol may involve scavenging of $HO^\bullet$, tyrosyl radical or $NO_2^\bullet$. All of these reactions are thermodynamically feasible when considering the reduction potential of the redox couples involved: ubisemiquinone/ubiquinol (+0.19 V; [34]), or $HO^\bullet/H_2O$ (2.3 V; [35]), $Tyr^\bullet Tyr$ (0.93 V; [36,37]), and $NO_2^\bullet/NO_2^-$ (+0.99 V; [38]).

In summary, a cohort of reactions involving mitochondrial ubiquinol with nitrogen-centred radicals have consequences for mitochondrial integrity and function and, most likely, for cellular effect signalled by mitochondrial changes. The implications of this redox network should be viewed primarily in terms of O_2 and $^\bullet NO$ gradients, which establish a dynamic interplay between $^\bullet NO$ metabolism, production of oxyradicals, regulation of O_2 uptake and scavenging of $ONOO^-$.

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