

Quinone analogues regulate mitochondrial substrate competitive oxidation

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Abstract

Quinone derivatives are among the rare compounds successfully used as therapeutic reagents to fight mitochondrial diseases. However, their beneficial effect appears to depend on their side chain which presumably governs their interaction with the respiratory chain. The effect of four quinone derivatives was comparatively studied on NADH- and succinate-competitive oxidation by a sub-mitochondrial fraction. Under our experimental conditions, the less hydrophobic derivatives (menadione, duroquinone) poorly affected electron flow from either NADH or succinate to oxygen, yet readily diverting electrons from isolated complex I. This latter effect was abolished by succinate addition. More hydrophobic derivatives (idebenone, decylubiquinone) stimulated oxygen uptake from succinate. But while NADH oxidation was slightly inhibited by idebenone, it was somewhat increased by decylubiquinone. As a result, idebenone strongly favoured succinate over NADH oxidation. This study therefore suggests that any therapeutic use of quinone analogues should take into account their specific effect on each respiratory chain dehydrogenase.

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Major advances have been made in the identification of the molecular bases and our understanding of mitochondrial oxidative phosphorylation (OXPHOS) diseases [1,2]. Yet, the management of patients is essentially supportive [2]. Indeed, the consequences of OXPHOS defects are poorly understood and, except for rare cases of coenzyme Q₁₀ (CoQ₁₀) [3,4] depletion or Friedreich ataxia [5], there is no advisable treatment. Nevertheless, either CoQ₁₀ or its derivatives have been given to a number of patients presenting an OXPHOS defect, with a mitigated effect on the clinical condition of the patients [6].

In mitochondria, CoQ₁₀ is a lipid component found in a large excess compared to any other electron carrier present in the inner membrane [7,8]. It transfers electrons from the various dehydrogenases to respiratory chain complex III [9]. Its redox status controls the equilibrium between the reducing activities of the dif-

ferent dehydrogenases. Notably a high level of CoQ₁₀ reduction is known to variably reduce the activity of all the dehydrogenases but the succinate dehydrogenase (SDH) which is upregulated under reducing condition [10,11].

Based on these considerations, we decided to study the effect of exogenous quinones on the activities of complexes I and II, and their equilibrium during the co-oxidation of their respective substrate, i.e., NADH and succinate. Unfortunately, CoQ₁₀ turned out to be too hydrophobic to be confidently handled, and we therefore used four of its derivatives, i.e., menadione, duroquinone, idebenone, and decylubiquinone. Menadione and idebenone have both been used to fight mitochondrial diseases [12,13]. However, the former, prone to autooxidation, can readily induce oxidative damages and has serious side effects; a toxicity which has been proposed to be used as a tool to get rid of cancer cells [14].

Our results show that quinone analogues have quite contrasting effects on the respiratory chain dehydrogenase activities.

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Materials and methods

Sub-mitochondrial fraction. Liver from control mice (multiple crossings between D57BL/6 and 129Sv strains) was homogenized at 4°C in 5 ml buffer consisting of 250 mM saccharose, 2 mM EGTA, 40 mM KCl, 20 mM Tris-HCl (pH 7.2), and bovine serum albumin (BSA; 1 mg/ml) [15]. The homogenate was filtered through a nylon net (90 µm mesh) and centrifuged (2000g, 5 min) to eliminate the heavy cell debris. The supernatant was stored at -80°C. This frozen supernatant, enriched in sub-mitochondrial fractions (SMF), was used for the comparative measurement of oxygen uptake, succinate and NADH consumption. A crude SMF-enriched preparation had to be used in this study as any purification procedure of these particles resulted in a drastic loss of poorly bound cytochrome *c*. Consequently, the potential incidence of contaminating activities was carefully taken into consideration and it was shown to be mostly negligible in the context of this study, thanks to the use of specific inhibitors.

Enzyme measurements. All measurements were carried out on SMF (final concentration 400 µg protein/ml) in 0.3 M mannitol, 10 mM KH_2PO_4 (pH 7.4), 10 mM KCl, 5 mM MgCl_2 , and 1 mg/ml BSA. The oxidation of NADH by the rotenone-sensitive complex I was spectrofluorometrically studied (excitation 378 nm, 10 nm bandpass; emission 455 nm, 5 nm bandpass; Perkin-Elmer LS-50B Spectrofluorimeter) [16]. The reduction of exogenous quinone derivatives by the malonate-sensitive succinate dehydrogenase was spectrophotometrically estimated by the reduction of dichlorophenolindophenol at 600 nm (SLM-Aminco DW-2000 UV-VIS spectrophotometer) [15]. The electron transfer from NADH (NADH- O_2 oxidoreductase) and from succinate to oxygen (succinate- O_2 oxidoreductase) was polarographically studied (Hansatech Instrument) as described [15]. Spectrophotometric, spectrofluorimetric, and polarographic measurements were all carried out at 37°C in magnetically stirred cells.

We tested a range of 5 concentrations (0, 20, 40, 60, and 80 µM) of four exogenous quinone derivatives (menadione, duroquinone, decylubiquinone, and idebenone). The final concentrations of succinate (10 mM) and NADH (80 µM) were saturating concentrations ensuring linear activities during the duration of the assays. The concentrations of the specific inhibitors were 10 mM and 4 µM for malonate (inhibitor of the complex II) and rotenone (inhibitor of complex I), respectively. The experiment duration was similar whatever the device and the assay considered and the indicated values are means \pm 1 SD from 5 to 6 experiments. In order to study mitochondrial substrate competitive oxidation, substrates were subsequently added at 2 min interval time, allowing to estimate both the specific oxidation rate of the first substrate (either succinate or NADH) and the competitive oxidation rate in the presence of the second substrate. When quantifying the effect of exogenous quinone derivatives, these latter were added 2 min after the first substrate addition (either succinate or NADH), followed by the addition of the second substrate (either succinate or NADH) 2 min later. This permits to estimate the effect of the various quinone derivatives on both the specific oxidation of each substrate and their competitive oxidation.

Quinone analogue quantification. Quinone analogue content was determinate in SMF (20 µl) after 5 min incubation followed by lipid extraction using chloroform/methanol/ H_2O (2/1/1). After centrifugation (10,000g, 5 min at 25°C), the pellet was washed with 1 ml PBS and resuspended in 1 ml ethanol. Extinction coefficients, estimated between 250 and 350 nm for each quinone analogue using successive dilutions in ethanol, were 22.1, 8.6, 7.9, and 7.9 $\text{mM}^{-1} \text{cm}^{-1}$ for menadione, duroquinone, idebenone, and decylubiquinone, respectively.

Reagents. Stock solutions of quinone derivatives (30 mM) were prepared in dimethyl sulfoxide. Idebenone was a kind gift from Takeda Chemical Company (Japan). All other chemicals were analytical grade reagents purchased from Sigma-Aldrich (France).

Results

Substrate oxidation by SMF

Comparison of the rates of NADH fluorescence decay (33 ± 2 nmol/min/mg prot) and NADH-dependent oxygen uptake (16 ± 1 nmol/min/mg prot) indicated that electrons were only used for a divalent reduction of oxygen. The full rotenone sensitivity of the two processes indicated that electrons were exclusively transferred to oxygen through the mitochondrial respiratory chain complex I. Oxidation rate was linear for at least 10 min and limited by the residual cytochrome *c* associated with mitochondrial membranes in SMF as indicated by the increased oxidation observed upon cytochrome *c* addition (not shown). No evidence of either interfering non-mitochondrial NADH dehydrogenase activity or of significant monovalent reduction of oxygen (superoxide formation) could be obtained under these experimental conditions. Succinate oxidation was 22 ± 2 nmol/min/mg prot (i.e., about 130% of NADH oxidation rate) and fully abolished by the addition of malonate.

Effect of menadione

At the used concentrations (0–80 µM), menadione had no effect on the cytochrome *c*-limited electron transfer to oxygen by SMF, either from NADH or from succinate (Figs. 1A and B) or on the co-oxidation of these two substrates (Fig. 1C). Similarly, menadione did not affect the activity of isolated complex II (Fig. 2A). However, menadione significantly increased the activity of the NADH dehydrogenase of the SMF (spectrofluorometrically measured) (Fig. 3A). This stimulatory effect was reduced in the presence of succinate (Fig. 3B). At 20 µM menadione, the activity of the rotenone-sensitive NADH dehydrogenase was roughly brought back to its initial level (in the absence of succinate), i.e., a 40% decrease (comparison Figs. 3A and B; Men 20 µM). This suggests that increased reduction of the respiratory chain triggered by the succinate dehydrogenase exerted an inhibitory effect on the NADH dehydrogenase.

Effect of duroquinone

Similarly to menadione, duroquinone had no effect on the electron transfer from NADH to oxygen (Fig. 1A) and did not significantly change the activity of the succinate dehydrogenase when measured alone (Fig. 2B). Yet, it had a slight inhibitory effect on the activity of the succinate oxidase, about 20% at a concentration of 80 µM duroquinone (Fig. 1B). Similarly to menadione, it also activated the rotenone-sensitive NADH dehydrogenase but to a much lower extent (less than 20% for duroquinone versus about 60% for menadione).

Effect of decylubiquinone

Similar to idebenone, decylubiquinone has a 10 carbon side chain, but ended with a methyl instead of a hydroxyl group, conferring a higher hydrophobicity to this molecule. Results obtained with decylubiquinone were reminiscent of those obtained with idebenone. Decylubiquinone significantly activated the succinate dehydrogenase (Fig. 2D) and the succinate-O₂ oxidoreductase (Fig. 1B). However, in contrast with idebenone, it significantly activated NADH-O₂ oxidoreductase (around 20% at 80 μM decylubiquinone) (Fig. 1A) and the NADH dehydrogenase (Fig. 3A).

Because the only difference between idebenone and decylubiquinone is the presence of a terminal charge on the idebenone side chain, which predictably affects the interaction with mitochondrial membrane, we next estimated the respective amount of each studied quinone derivative sequestered in SMF (initial concentration 80 μM). Taking into account the differential yield of quinone homologue extraction under our experimental condition (menadione, duroquinone, about 50%; idebenone, decylubiquinone, 100%), it appears that neither menadione nor duroquinone could be detected in the SMF pellet, while 1–2% (about 50–100 μM) of initially added idebenone could be measured. Finally, ten times more decylubiquinone (in the milli-molar order), the most apolar compound, was found in the SMF pellet. The quite different amounts of sequestered quinone might obviously account for part of their differential effect.

Finally, both decylubiquinone and idebenone strongly affected the oxidation of NADH during its co-oxidation with succinate (comparison of Figs. 3A and B). While decylubiquinone-induced NADH dehydrogenase activation was practically abolished, no rotenone-sensitive NADH oxidation could be measured under these conditions in the presence of 60–80 μM idebenone (Fig. 3B).

Discussion

The scope of this study was to examine the effect of exogenous quinones on mitochondrial substrate competitive co-oxidation. In a recent and detailed kinetic study, several quinone derivatives have been shown to differentially divert electron flow from respiratory chain complex I [17]. Based on this observation, we selected four quinone derivatives and studied their effect on the co-oxidation of NADH and succinate. These derivatives mostly differ by their hydrophobicity. Menadione, a naphthoquinone derivative, and duroquinone, a quinone ring substituted with 4 methyl groups, are much less hydrophobic than decylubiquinone and idebenone, which have a 10 carbon-side chain differing by the ter-

minal substitutes only, a methyl or a hydroxyl group, respectively.

We found that both exogenous menadione and duroquinone did not significantly affect the balance between NADH and succinate oxidations. In contrast, the 10 carbon side chain derivatives, decylubiquinone and idebenone, significantly affected this balance, favouring succinate over NADH oxidation. It has been previously established that a partial deficiency of complex I results in a full blockade of NADH oxidation in co-oxidation experiments with succinate [16]. In keeping with this, it can be predicted that providing exogenous quinones such as decylubiquinone or idebenone could be detrimental in case of complex I deficiency by further favouring complex II activity. Conversely, their use could be rather beneficial in case of complex II deficiency.

Noticeably, although the two analogues only vary by the terminal group of their side chain (a hydroxyl versus a methyl group for decylubiquinone and idebenone, respectively), their effects on NADH oxidation alone strongly differ [17]. We found that the partition of idebenone and decylubiquinone in SMF was also strongly different. Based on this observation, it can be suggested that the more hydrophobic decylubiquinone could better convey electrons to the hydrophobic CoQ₁₀ naturally present in the mitochondrial membrane, hence maintaining an oxidized micro-environment favouring complex I activity. In contrast, idebenone, because of its poor hydrophobicity, would slowly react with CoQ₁₀ and stay under a reduced form, creating an unfavourable micro-environment for the NADH dehydrogenase activity.

Noticeably, beside their effect on the electron flow balance between the RC dehydrogenases, quinone derivatives under a reduced form can be useful as potent antioxidant molecules [18]. In keeping with this, idebenone, prone to create a reduced micro-environment around mitochondrial membrane, should be a favourable quinone analogue. This is indeed illustrated by the successful use of idebenone to counteract the life-threatening cardiomyopathy in Friedreich ataxia, simultaneously restoring superoxide-sensitive enzyme activities [19].

Ultimately, the *in vivo* effect of quinone derivatives presumably results from (i) their biodisponibility, (ii) their targeting to the subcellular defective compartment, i.e., the mitochondria in the context of mitochondrial diseases, (iii) their antioxidant properties, and (iv) their specific effect on respiratory chain dehydrogenase activities. Obviously, none of the derivatives can exactly fulfil CoQ₁₀ function in the mitochondrial membrane. Although restricted to only two of the mitochondrial dehydrogenases (complexes I and II), our study emphasized the amplified effect of quinone derivatives on dehydrogenases when studied during substrate co-oxidation. It also shows the importance of a judicious

selection of quinone derivatives when attempting to either improve or balance electron flow, or to act as an anti-oxidant in a defective respiratory chain.

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References

- [1] S. DiMauro, E.A. Schon, Mitochondrial respiratory-chain diseases, *N. Engl. J. Med.* 348 (2003) 2656–2668.
- [2] P.F. Chinnery, D.M. Turnbull, Epidemiology and treatment of mitochondrial disorders, *Am. J. Med. Genet.* 106 (2001) 94–101.
- [3] A. Rotig, E.L. Appelkvist, V. Geromel, D. Chretien, N. Kadhon, P. Edery, M. Lebideau, G. Dallner, A. Munnich, L. Ernster, P. Rustin, Quinone-responsive multiple respiratory-chain dysfunction due to widespread coenzyme Q10 deficiency, *Lancet* 356 (2000) 391–395.
- [4] O. Musumeci, A. Naini, A.E. Slonim, N. Skavin, G.L. Hadjigeorgiou, N. Krawiecki, B.M. Weissman, C.Y. Tsao, J.R. Mendell, S. Shanske, D.C. De Vivo, M. Hirano, S. DiMauro, Familial cerebellar ataxia with muscle coenzyme Q10 deficiency, *Neurology* 56 (2001) 849–855.
- [5] A.O. Hausse, Y. Aggoun, D. Bonnet, D. Sidi, A. Munnich, A. Rotig, P. Rustin, Idebenone and reduced cardiac hypertrophy in Friedreich's ataxia, *Heart* 87 (2002) 346–349.
- [6] V. Geromel, N. Darin, D. Chretien, P. Benit, P. DeLonlay, A. Rotig, A. Munnich, P. Rustin, Coenzyme Q(10) and idebenone in the therapy of respiratory chain diseases: rationale and comparative benefits, *Mol. Genet. Metab.* 77 (2002) 21–30.
- [7] A. Kroger, M. Klingenberg, The kinetics of the redox reactions of ubiquinone related to the electron-transport activity in the respiratory chain, *Eur. J. Biochem.* 34 (1973) 358–368.
- [8] M. Turunen, J. Olsson, G. Dallner, Metabolism and function of coenzyme Q, *Biochim. Biophys. Acta* 1660 (2004) 171–199.
- [9] A. Tzagoloff, *Mitochondria*, Plenum Press, New York, 1982.
- [10] M. Gutman, E.B. Kearney, T.P. Singer, Regulation of succinate dehydrogenase activity by reduced coenzymes Q10, *Biochemistry* 10 (1971) 2726–2733.
- [11] P. Rustin, A. Munnich, A. Rotig, Succinate dehydrogenase and human diseases: new insights into a well-known enzyme, *Eur. J. Hum. Genet.* 10 (2002) 289–291.
- [12] D. Mowat, D.M. Kirby, K.R. Kamath, A. Kan, D.R. Thorburn, J. Christodoulou, Respiratory chain complex III [correction of complex] in deficiency with pruritus: a novel vitamin responsive clinical feature, *J. Pediatr.* 134 (1999) 352–354.
- [13] P. Rustin, J.C. von Kleist-Retzow, K. Chantrel-Groussard, D. Sidi, A. Munnich, A. Rotig, Effect of idebenone on cardiomyopathy in Friedreich's ataxia: a preliminary study, *Lancet* 354 (1999) 477–479.
- [14] J. Verrax, J. Cadrobbi, M. Delvaux, J.M. Jamison, J. Gilloteaux, J.L. Summers, H.S. Taper, P. Buc Calderon, The association of vitamins C and K3 kills cancer cells mainly by autophagy, a novel form of cell death. Basis for their potential use as adjuvants in anticancer therapy, *Eur. J. Med. Chem.* 38 (2003) 451–457.
- [15] P. Rustin, D. Chretien, T. Bourgeron, B. Gerard, A. Rotig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, *Clin. Chim. Acta* 228 (1994) 35–51.
- [16] V. Geromel, B. Parfait, J.C. von Kleist-Retzow, D. Chretien, A. Munnich, A. Rotig, P. Rustin, The consequences of a mild respiratory chain deficiency on substrate competitive oxidation in human mitochondria, *Biochem. Biophys. Res. Commun.* 236 (1997) 643–646.
- [17] M.L. Genova, M.M. Pich, A. Biondi, A. Bernacchia, A. Falasca, C. Bovina, G. Formiggini, G.P. Castelli, G. Lenaz, Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant, *Exp. Biol. Med. (Maywood)* 228 (2003) 506–513.
- [18] L. Ernster, G. Dallner, Biochemical, physiological and medical aspects of ubiquinone function, *Biochim. Biophys. Acta* 1271 (1995) 195–204.
- [19] P. Rustin, D. Bonnet, A. Rötig, A. Munnich, D. Sidi, Idebenone restores mitochondrial respiratory chain enzyme activities in the cardiac muscle in Friedreich's ataxia, *Neurology* 62 (2004).