Redox regulation of mitochondrial functional activity by quinones

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Quinones are among the rare compounds successfully used as therapeutic agents to correct mitochondrial diseases and as specific regulators of mitochondrial function within cells. The aim of the present study was to elucidate the redox-dependent effects of quinones on mitochondrial function. The functional parameters [respiratory activity, membrane potential, and reactive oxygen species (ROS) generation] of isolated rat liver mitochondria and mitochondria in intact cells were measured in the presence of eight exogenously applied quinones that differ in lipophilicity and one-electron reduction potential. The quinones affected the respiratory parameters of mitochondria, and dissipated the mitochondrial membrane potential as well as influenced (either decreased or enhanced) ROS generation, and restored the electron flow during electron transport chain inhibition. The stimulation of ROS production by juglone and 2,5-di-tert-butyl-1,4-benzoquinone was accompanied by a decrease in the acceptor control and respiration control ratios, dissipation of the mitochondrial membrane potential and induction of the reverse electron flow under succinate oxidation in isolated mitochondria. Menadione and 2,3,5-trimethyl-1,4-benzoquinone, which decreased the mitochondrial ROS generation, did not affect the mitochondrial potential and, *vice versa*, were capable of restoring electron transport during Complex I inhibition. In intact C6 cells, all the quinones, except for coenzyme Q_{10}, decreased the mitochondrial membrane potential. Juglone, 1,4-benzoquinone, and menadione showed the most pronounced effects. These findings indicate that quinones with the reduction potential values $E_{1/2}$ in the range from -99 to -260 mV were effective redox regulators of mitochondrial electron transport.

Keywords: quinone, mitochondria, respiration, membrane potential, reactive oxygen species, one-electron reduction potential

Introduction

Quinones are oxidized derivatives of aromatic compounds (such as benzene or naphthalene) stabilized by conjugation (Fig. 1). Electron-donating substituents, such as phenols and catechols, increase the nucleophilicity of the ring and contribute to high redox potential of the quinones. Some quinones serve as electron acceptors in electron transport chains (ETCs), such as those participating in photosynthesis (plastoquinone, phylloquinone), and aerobic respiration (ubiquinone). Quinones are capable of regulating cell functions, inducing reactive oxygen species (ROS) generation or, *vice versa*, manifesting antioxidant properties (in a reduced form). Another mechanism for quinone regulation of cellular properties is arylation

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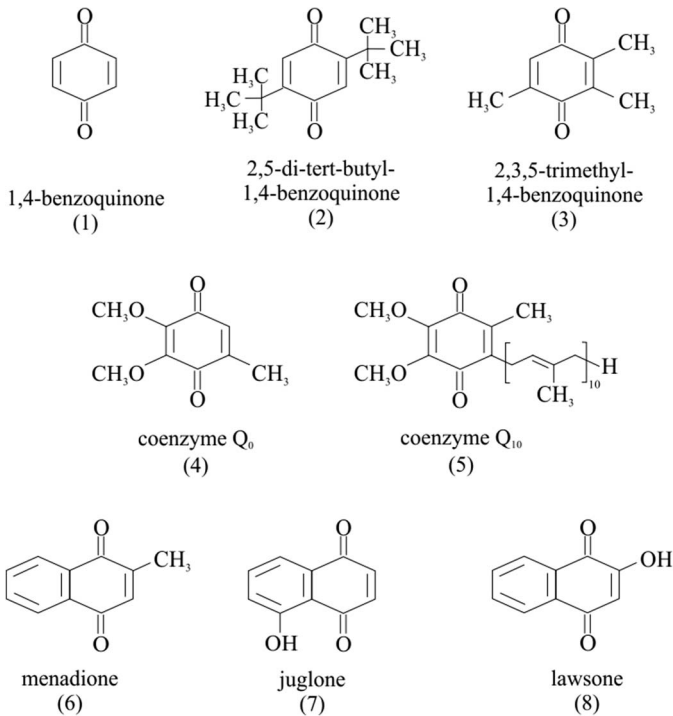Phone: +375 152 484583; Fax: +375 152 434121; E-mail: zavodnik_il@mail.ru

Fig. 1. Structure of the quinones used

of SH and NH_2 groups of some functionally significant proteins (6, 39, 49, 50, 55). The rate and direction of oxidation–reduction reactions are determined by quinone reduction potentials. Quinones with one-electron reduction potential ($E_{1/2}$) values in the range from −240 to −170 mV and from −50 to 25 mV are effective redox cyclic agents and induce oxidative stress in biological systems (14, 43, 50). Quinones can be reduced according to one- or two-electron mechanisms by intracellular reductases, including mitochondrial NADH:ubiquinone oxidoreductase, and the mitochondrial isoform of DT-diaphorase (NAD(P)H: quinone oxidoreductase 1 (NQO1) (29, 38–40). Quinones can recapture electrons from ubiquinol of the mitochondrial ETC and influence the electron flow between ETC dehydrogenases (5, 10, 11, 31). Quinone's ability to interact with ETC components depends on the lipophilic properties of a quinone (5, 11, 31).

Mitochondria are the major cellular sites of ROS production. Earlier studies have suggested that Complexes I (producing $O_2^{\bullet-}$ to the matrix) and III (producing $O_2^{\bullet-}$ to both the matrix and intermembrane space) of the mitochondrial ETC are the main sites of the ROS generation (4). Generated by mitochondria, ROS act as redox signals in triggering cellular events such as apoptosis, proliferation, and senescence (35) and quinones, depending on their structure, affect mitochondrial ROS generation. It was shown that 1,4-benzoquinone, 1,4-naphthoquinone, and their derivatives decreased the mitochondrial membrane potential in cells and calcium-loaded isolated mitochondria. This effect is related to mitochondrial permeability transition pore opening caused by quinone-induced ROS generation and/or by arylation of SH― groups of proteins participating in pore formation (5, 20, 21, 24, 26, 41, 51). Menadione, duroquinone, and coenzyme Q_1 are capable of recovering respiratory and phosphorylating mitochondrial functions shunting ETC complexes during rotenone

Table I. One-electron reduction potential ($E_{1/2}$) (7, 38) and partition coefficient in octanol/water system (log$P$) (53) for the quinones studied

	Quinones	log$P$	$E_{1/2}$, mV
1	BQ	0.2	+78
2	DTBBQ	3.4	−260
3	TMBQ	1.8	−165
4	Coenzyme Q_0	0.8	−110
5	Coenzyme Q_{10}	19.4	−230[a]; −36[b]
6	Menadione	2.2	−203
7	Juglone	1.9	−99
8	Lawsone	0.9	−415

[a]Theoretically calculated value (52); [b]experimental value measured for submitochondrial particles from beef-heart mitochondria (15)

inhibition of respiration. Being reduced by mitochondrial DT-diaphorase, these quinones either can transfer electrons directly to Complex III, coenzyme Q, or they can play a role of succinate dehydrogenase substrates (9, 10, 13, 23, 37).

Quinone derivatives are among rare compounds successfully used as therapeutic agents to treat mitochondrial diseases (5, 9) and bypass the damaged sites in mitochondria. To reveal the dependence of quinone mitochondrial effects on their physicochemical properties, the group of quinones varying in lipophilicity and one-electron reduction potential $E_{1/2}$ was studied. We elucidated the effects of eight added quinones: 5-hydroxy-1,4-naphthoquinone (juglone), 1,4-benzoquinone (BQ), 2,5-di-tert-butyl-1,4-benzoquinone (DTBBQ), 2-methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone), coenzymes Q_0 and Q_{10}, and 2,3,5-trimethyl-1,4-benzoquinone (TMBQ) (Fig. 1) on the respiratory parameters, mitochondrial membrane potential, and ROS production by isolated rat liver mitochondria. The partition coefficients in the octanol/water system (log$P$) and $E_{1/2}$ of quinones used in this study are represented in Table I.

Materials and Methods

Chemicals

Succinic acid disodium salt hexahydrate, L-glutamic acid sodium salt, maleic acid disodium salt, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), menadione, coenzyme Q_0, coenzyme Q_{10}, lawsone, juglone, rotenone, amytal, 3,3′-methylene-bis-(4-hydroxycoumarin) (dicumarol), and ADP were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and the fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), JC-1, and MitoSOX Red were purchased from Molecular Probes, Inc. (USA). BQ, TMBQ, and DTBBQ were synthesized and purified, as described by Flaig et al. (19); Fieser and Fieser (18); and Adams et al. (1). All other reagents were purchased from POCh (Gliwice, Poland) and Reakhim (Moscow, Russia) and were of analytical grade. All solutions were made with water purified in the Milli-Q system.

Quinones were dissolved in dimethyl sulfoxide (DMSO). The final DMSO content in the probe did not exceed 0.1%.

Rat liver mitochondria: Isolation and respiration measurements

Mitochondria were isolated by the standard procedure of differential centrifugation from the rat liver (27). The liver was quickly removed and placed into the ice-cold isolation medium containing 250 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, pH 7.2. The tissue was cut into small pieces and homogenized in a glass-Teflon homogenizer with isolation medium (at 2 °C). The homogenate was centrifuged at $600 \times g$ for 10 min, and the supernatant was centrifuged at $8,500 \times g$ for 10 min. The obtained pellet was washed in buffer containing 250 mM sucrose, 5 mM Tris–HCl, and pH 7.2 (at 2 °C). The mitochondrial pellet was resuspended in the buffer to an approximate protein concentration of 35–40 mg/ml. The protein concentration was determined as described by Lowry et al. (34). Animals were killed according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the Bioethics Committee of the Institute for Biochemistry of Biologically Active Compounds of the National Academy of Sciences of Belarus (registration number 4-axd dated March 18, 2014).

The respiration of mitochondria was measured using a laboratory-made oxygen Clark-type electrode and a hermetic polarographic cell (volume 1.25 ml) with constant gentle stirring (16). The electrode was calibrated by bubbling the polarographic cell with pure nitrogen ($pO_2 = 0$) and atmospheric air (atmospheric pO_2). The oxygen consumption by the electrode was negligible. The incubation medium contained 125 mM KCl, 50 mM sucrose, 10 mM Tris–HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, with 0.5 mM EDTA (EDTA containing) or without EDTA (EDTA-free medium), pH 7.4. The experiments were performed at 25 °C using 5 mM L-glutamate and 2 mM malate mixture (Glu + Mal) as respiratory substrates. Mitochondrial protein concentration in the sample was 1.0 mg/ml. The functional activity of mitochondria was determined by the acceptor control ratio (ACR), equal to the ratio of the respiratory rates (V_3/V_2) of mitochondria in States 3 and 2, the respiration control ratio (RCR = V_3/V_4) and the coefficient of phosphorylation (ADP/O) (the ratio of the amount of ADP added to the amount of oxygen consumed throughout phosphorylation, characterizing thermodynamic efficiency of oxygen consumption). State 1 corresponded to the respiration of mitochondria oxidizing endogenous substrates (basal respiration, V_1). State 2 corresponded to the respiration in the presence of the substrate added (V_2). The rate of mitochondrial respiration corresponding to State 3 (V_3) was recorded after addition of 180 μM ADP. State 4 (V_4) corresponded to the respiration after ADP depletion. Oxygen consumption in each metabolic state was measured following 5 min after the corresponding additions (substrates or ADP) have been made. We expressed the mitochondrial respiration rates in the presence of the quinones as percentage of the control value (V_1^n, V_2^n, V_3^n). We also used the parameter $<V> = (V_1^n + V_2^n)/2$ removed by averaging the basal and substrate-dependent respiration rates.

Cell culture

C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). The cultures were incubated at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. After the cells reached 70% confluency, they were harvested, washed

with Hepes buffer (131 mM NaCl, 5 mM KCl, 1.3 mM CaCl$_2$, 1.3 mM MgSO$_4$, 6 mM glucose, 20 mM Hepes, pH 7.3), resuspended up to a final density of 1×10^6 cells/ml and used for experiments.

Mitochondrial membrane potential
The mitochondrial membrane potential ($\Delta\psi$) of isolated mitochondria was measured in the medium (0.2 M sucrose, 20 mM Tris–HCl, 2.5 mM MgSO$_4$, 2.5 mM KH$_2$PO$_4$, 20 mM KCl, 1 mM EDTA, pH 7.2) at 27 °C fluorometrically using fluorescent probe safranin O (2). The excitation wavelength was 495 nm (slit 3 nm) and the emission wavelength was 586 nm (slit 3 nm) (an SM 2203 spectrofluorimeter, Solar, Belarus), the dye concentration was 8 μM and the mitochondrial protein concentration was 0.3 mg/ml. Isolated mitochondria were added to the media containing respiratory substrate (5 mM succinate or 5 mM L-glutamate and 2 mM malate mixture). At the end of the measurements, uncoupler FCCP (0.5 μM) was added to the mitochondria to achieve a complete depolarization. The membrane potential values (mV) were determined using a calibration plot, which represented the dependence of the safranin O fluorescence intensity on the membrane potential value, according to the Nernst equation:

$$\Delta\psi = 60 \log \frac{[\text{K}^+]_{\text{out}}}{[\text{K}^+]_{\text{in}}} (\text{mV}),$$

where $[\text{K}^+]_{\text{in}}$ is the intramitochondrial potassium concentration (120 mM) and $[\text{K}^+]_{\text{out}}$ is the extramitochondrial potassium concentration in the media that varies from 0 to 20 mM in the presence of ionophore valinomycin (0.28 μM) (36).

Mitochondrial energization in intact C6 glioma cells was evaluated using mitochondrial membrane potential sensitive carbocyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanine iodide) (44). This lipophilic and cationic dye accumulates within mitochondria driven by the negative membrane potential and forms concentration-dependent J-aggregates. When excited at 490 nm, the monomeric form of the dye emits at 527 nm, while the J-aggregates show maximum emission at 590 nm. Briefly, 1×10^6 cells were treated with quinones for 20 min, washed with Hepes buffer, and incubated in the presence of 1 μM JC-1 for 15 min. After washing cells twice again with Hepes buffer, JC-1-related fluorescence was analyzed in a spectrofluorimeter (SM2203, Solar, Belarus).

ROS generation in mitochondria
The mitochondrial ROS generation after addition of quinones (1–10 μM) in the presence or absence of rotenone (1 μM) was estimated fluorometrically using fluorescent probe H$_2$DCF-DA and Glu+Mal as respiratory substrate. H$_2$DCF-DA was de-esterified preliminarily by incubation with 0.1 M NaOH for 30 min to form non-fluorescent H$_2$DCF (8). The H$_2$DCF solution was adjusted to pH 7.4 by addition of 0.1 M HCl. The stock concentration of the H$_2$DCF was 100 μM. Ten microliters of the prepared H$_2$DCF solution was added to 1 ml of the mitochondrial suspension (0.3 mg protein/ml). The extramitochondrial ROS were measured by oxidation of H$_2$DCF to fluorescent 2′,7′-dichlorofluorescein (DCF) (22, 56). The experiments were carried out at 37 °C and pH 7.4. The DCF fluorescence intensity ($\lambda_{\text{ex}} =$ 488 nm, $\lambda_{\text{em}} = 530$ nm) was recorded by a spectrofluorimeter for 40 min.

For studying of mitochondrial superoxide production in C6 cells, MitoSOX Red [fluorogenic dye specifically targeted to mitochondria in live cells (45)] was added to 1.25-μM final concentration for 20 min, after which the cells were washed twice with Hepes

buffer and analyzed. Fluorescence intensity of MitoSOX ($\lambda_{ex} = 510$ nm, $\lambda_{em} = 580$ nm) was measured using a spectrofluorimeter at 0, 10, 20, 30, and 40 min intervals after quinone addition to cell suspension.

Statistical analysis

The results were expressed as the means of four to five replicates $\pm$ SD and the statistical analysis was conducted using the analysis of variance. We used the standard unpaired Student's t-test and nonparametric Spearman's rank correlation for the comparison of the raw and transformed data showing no departures from normality (according to Shapiro–Wilk's test). $p < 0.05$ was taken to indicate statistical significance. Calculations were performed using the statistical software packages StatSoft Statistica 6.0 and GraphPad Prism 4.0. Correlation analysis was performed utilizing the GraphPad Prism 4.0. The fluorescent kinetic curves were typical for three to four independent experiments performed.

Results

Effects of quinones on the respiratory activity of mitochondria

We examined the respiratory activities of isolated rat liver mitochondria exposed to quinones of different chemical structures (Fig. 1). As Tables II and III show, the rate of basal oxygen consumption (V_1), in the case of Glu+Mal as a respiratory substrate, increased twofold in the presence of DTBBQ, coenzyme Q_0, and menadione (10 µM). Simultaneously, DTBBQ decreased the ADP-stimulated respiration V_3 by 30% ($p < 0.05$) without significant changes in the respiration rates V_2 and V_4. Coenzyme Q_0 did not considerably influence the respiratory

Table II. Respiratory activity of rat liver mitochondria on exposure to quinones (10 µM) during Glu+Mal oxidation

	Basal respiration rate (V_1^n), %	Glu+Mal-dependent respiration rate (V_2^n), %	ADP-stimulated respiration rate (V_3^n), %	Respiration rate after ADP consumption (V_4^n), %	$\langle V \rangle = \left(\frac{V_1^n + V_2^n}{2} \right)$, %
Control	100	100	100	100	100
BQ	121 ± 30	104 ± 23	103 ± 15	94 ± 31	113
DTBBQ	$217 \pm 47^*$	90 ± 14	$71 \pm 17^*$	95 ± 20	154
TMBQ	135 ± 46	98 ± 15	102 ± 10	100 ± 13	117
Coenzyme Q_0	$228 \pm 40^*$	77 ± 30	98 ± 18	100 ± 10	153
Coenzyme Q_{10}	128 ± 38	67 ± 45	94 ± 21	$62 \pm 35^*$	97
Menadione	$232 \pm 47^*$	$159 \pm 35^*$	111 ± 27	$188 \pm 35^*$	195
Juglone	$65 \pm 20^*$	$231 \pm 50^*$	$79 \pm 10^*$	$302 \pm 70^*$	148
Lawsone	100 ± 28	67 ± 34	106 ± 17	$63 \pm 23^*$	83

*Statistically significant in comparison with control, $p < 0.05$

Table III. ACR, RCR, and phosphorylation coefficient of rat liver mitochondria on exposure to quinones (10 μM) during Glu + Mal oxidation

	ACR (V_3/V_2)	RCR (V_3/V_4)	ADP/O
Control	4.3 ± 1.1	4.7 ± 1.8	2.7 ± 0.4
BQ	4.1 ± 1.5	3.1 ± 1.7	2.1 ± 0.8
DTBBQ	3.7 ± 1.2	$2.7 \pm 1.0*$	2.5 ± 0.7
TMBQ	4.4 ± 1.5	3.6 ± 0.5	2.6 ± 0.3
Coenzyme Q_0	6.6 ± 1.1	4.2 ± 1.0	3.0 ± 0.7
Coenzyme Q_{10}	$7.3 \pm 1.2*$	$6.3 \pm 0.8*$	3.0 ± 0.7
Menadione	$2.8 \pm 0.2*$	$2.7 \pm 0.7*$	2.3 ± 0.4
Juglone	$1.8 \pm 0.5*$	$1.3 \pm 0.4*$	$1.2 \pm 0.8*$
Lawsone	$8.3 \pm 1.0*$	$7.2 \pm 1.0*$	2.5 ± 0.5

*Statistically significant in comparison with control, $p < 0.05$

rates V_2, V_3, and V_4, whereas menadione significantly enhanced the respiration rate V_2 (by 60%, $p < 0.05$) and V_4 (by 90%, $p < 0.05$), unchanging the rate V_3. Juglone caused a decrease of the V_1 (by 35%, $p < 0.05$) and V_3 respiration rates, but significantly elevated the rates of Glu+Mal-stimulated respiration (V_2, by 130%, $p < 0.05$) and respiration after ADP depletion (V_4, by 200%, $p < 0.05$). BQ and TMBQ did not change the respiration significantly. Coenzyme Q_{10} and lawsone decreased the respiration rate V_4 (by 40%, $p < 0.05$) and did not change the rates V_1, V_2, and V_3. The values for ACR and RCR were considerably increased in the presence of coenzyme Q_{10} and lawsone (1.3–1.7-fold, $p < 0.05$) (Table III). The phosphorylation coefficient (ADP/O) was unchanged after addition of these quinones. During mitochondrial exposure to menadione or juglone, the ACR was observed to decrease 1.5- and 2.4-fold ($p < 0.05$), respectively, whereas the RCR was found to diminish 1.7-fold ($p < 0.05$) for DTBBQ or menadione and 3.6-fold ($p < 0.05$) for juglone. Juglone most pronouncedly lowered the ACR, RCR, and ADP/O values. These data indicate that juglone is an effective uncoupling agent of mitochondrial respiration.

Quinone restoration of mitochondrial respiration during inhibition
It was shown earlier that menadione, idebenone, coenzyme Q_0, and coenzyme Q_1, but not coenzyme Q_{10}, partially restored mitochondrial activity under conditions of impaired Complex I function (23, 37). In our experiments, addition of amytal (10 μM), Complex I inhibitor, caused significant impairment in mitochondrial respiratory activity (Tables IV and V). TMBQ and menadione stimulated the basal, Glu+Mal-dependent, and ADP-stimulated respirations and increased the values of ACR, RCR, and ADP/O ($p < 0.05$), restoring functions of the mitochondrial ETC during inhibition of Complex I by amytal. The protective effect of coenzyme Q_0 was less pronounced (Tables IV and V). The other quinones studied did not show any significant preventing effect during Complex I inhibition (data not shown). As Tables IV and V indicate, the addition of two-electron quinone reduction inhibitor (mitochondrial DT-diaphorase inhibitor), dicumarol, to mitochondria during inhibition of Complex I prevented restoration of mitochondrial respiratory activity by menadione

Table IV. Effect of quinones (10 μM) on Glu + Mal-stimulated respiratory activity of rat liver mitochondria during inhibition of Complex I by amytal (10 μM) in the absence and the presence of dicumarol

	Basal respiration rate (V_1^n), %	Glu + Mal-dependent respiration rate (V_2^n), %	ADP-stimulated respiration rate (V_3^n), %	Respiration rate after ADP consumption (V_4^n), %
Control	100	100	100	100
Amytal	81 ± 33	66 ± 22	40 ± 22	94 ± 37
Amytal + TMBQ	$136 \pm 15^*$	$106 \pm 15^*$	66 ± 13	97 ± 9
Amytal + menadione	$132 \pm 10^*$	$140 \pm 29^*$	$91 \pm 12^*$	$177 \pm 17^*$
Amytal + coenzyme Q_0	120 ± 10	99 ± 16	36 ± 16	104 ± 31
Addition of dicumarol (10 μM)				
Amytal + dicumarol	155 ± 11	158 ± 60	60 ± 10	168 ± 34
Amytal + dicumarol + TMBQ	110 ± 15	200 ± 74	54 ± 25	169 ± 45
Amytal + dicumarol+ menadione	180 ± 15	$249 \pm 60^*$	87 ± 22	$332 \pm 70^*$
Amytal + dicumarol + coenzyme Q_0	165 ± 12	160 ± 70	70 ± 15	150 ± 30

*Statistically significant in comparison with corresponding control without quinone, $p < 0.05$

(partially), coenzyme Q_0 and TMBQ (completely). Thus, the restoration of the respiratory chain functions by quinones was due to their conversions to the phenolic form.

Effect of quinones on mitochondrial membrane potential

We studied the effect of naphtho- and benzoquinones on the membrane potential of intact mitochondria and under Complex I inhibition. In our experiments, quinones, impairing mitochondria respiration, induced dissipation of the mitochondrial membrane potential. As Fig. 2 demonstrates, addition of juglone (10 μM) caused a slow partial decrease of the membrane potential from −200 mV (for the control) to −150 mV within 10–15 min of Glu +Mal oxidation and did not affect the membrane potential during succinate oxidation (Fig. 3), which indicates quinone interaction with Complex I. The effect of DTBBQ on the membrane potential was less pronounced (Δψ was decreased by 12 ± 3 mV, $p < 0.05$) during both Glu + Mal and succinate oxidation (Figs 2 and 3). Figures 2 and 3 show that lawsone, menadione, coenzyme Q_{10}, and TMBQ at a concentration of 10 μM did not influence the membrane potential under Glu+Mal or succinate oxidation. At the same time, as shown in Fig. 4, menadione and TMBQ (10 μM) restored the Glu+Mal-generated mitochondrial potential to –(200 ± 23) mV (completely), or −(150 ± 15) mV (partially), respectively, after rotenone addition. Rotenone dissipated Glu+Mal – but not succinate-generated membrane potential. The inhibitor was added after 200 s following the quinone injection (Figs 3 and 4). Surprisingly, lawsone also slightly prevented rotenone-induced potential loss (Fig. 4). Dicumarol inhibited quinone restoration of the mitochondrial membrane potential dissipated

Table V. Effects of quinones (10 µM) on ACR, RCR, and phosphorylation coefficient of rat liver mitochondria during inhibition of Complex I by amytal (10 µM) in the absence and the presence of dicumarol; Glu + Mal is respiratory substrate

	ACR (V_3/V_2)	RCR (V_3/V_4)	ADP/O
Control	4.3 ± 1.1	4.7 ± 1.8	2.7 ± 0.4
Amytal	1.7 ± 0.5	1.5 ± 0.5	1.7 ± 0.7
Amytal + TMBQ	2.6 ± 0.1*	2.7 ± 0.4*	2.1 ± 0.3*
Amytal + menadione	2.6 ± 0.3*	2.3 ± 0.6	2.9 ± 0.5*
Amytal + coenzyme Q_0	2.3 ± 0.6	1.9 ± 0.4	2.1 ± 0.6
Addition of dicumarol (10 µM)			
Amytal + dicumarol	1.8 ± 0.5	1.6 ± 1.0	0.9 ± 0.6
Amytal + dicumarol + TMBQ	2.3 ± 0.6	2.0 ± 0.7	1.2 ± 0.6
Amytal + dicumarol + menadione	1.7 ± 0.3	1.3 ± 0.6	2.0 ± 0.7*
Amytal + dicumarol + coenzyme Q_0	2.0 ± 0.6	1.7 ± 0.5	1.0 ± 0.7

*Statistically significant in comparison with corresponding control without quinone, $p < 0.05$

by rotenone: completely in the case of TMBQ and partially in the case of menadione. BQ did not affect the membrane potential in the absence of rotenone but enhanced the inhibitory effect of rotenone during Glu+Mal oxidation (Fig. 4). Similarly, juglone was found to enhance the action of rotenone, increasing the rate of mitochondrial membrane potential loss
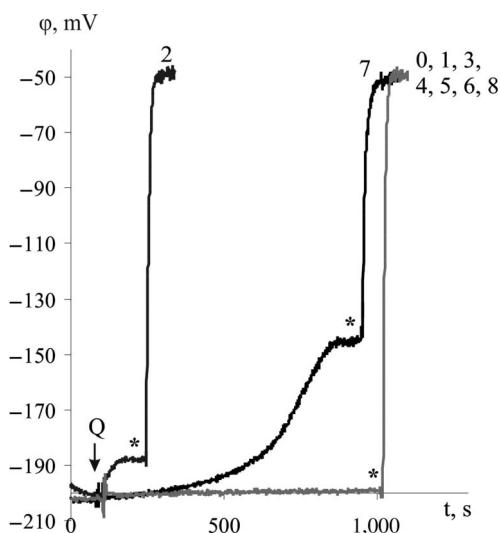

Fig. 2. Mitochondrial membrane potential in rat liver mitochondria exposed to 10 µM quinones with Glu +Mal as respiratory substrate: 0, control; 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone. Q and * designate moments of quinone and FCCP addition, respectively

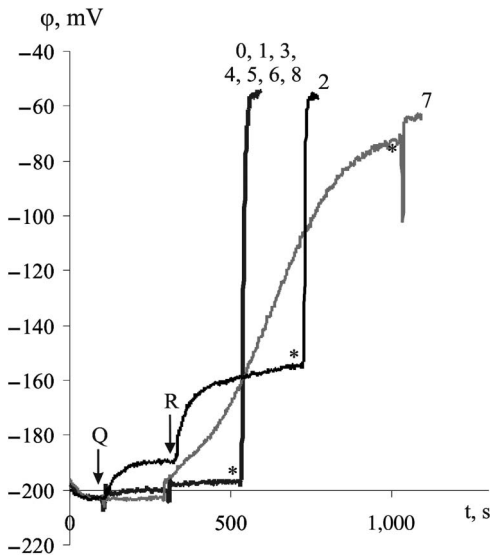*Fig. 3.* Mitochondrial membrane potential in rat liver mitochondria exposed to 10 μM quinones and rotenone (1 μM) with succinate as respiratory substrate: 0, control; 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone. Q, R, and * designate moments of quinone, rotenone and FCCP addition, respectively

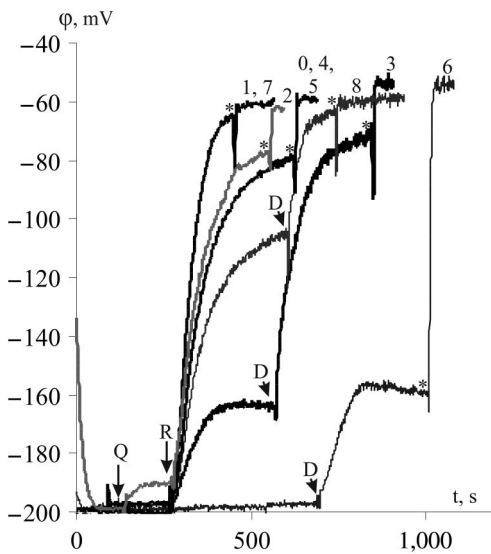*Fig. 4.* Mitochondrial membrane potential in rat liver mitochondria exposed to 10 μM quinones, rotenone (1 μM) and dicumarol (1 μM) with Glu+Mal as respiratory substrate: 0, control; 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone. Q, R, D, and * designate moments of quinone, rotenone, dicumarol, and FCCP addition, respectively

(Fig. 4). DTBBQ, coenzyme Q_0, and coenzyme Q_{10} did not prevent the inhibitory effect of rotenone. Figure 3 shows that rotenone did not change the membrane potential in succinate-oxidizing mitochondria but considerably dissipated the succinate-generated potential in the presence of juglone or DTBBQ. The combined action of DTBBQ and rotenone decreased the membrane potential during succinate oxidation by 45 ± 9 mV, $p < 0.05$, and the combined action of juglone and rotenone induced a complete loss of the potential. We suggested that juglone or DTBBQ catalyzed the reverse electron flow from Complex II to Complex I and, thus, in this way participated in the generation of the succinate-dependent membrane potential.
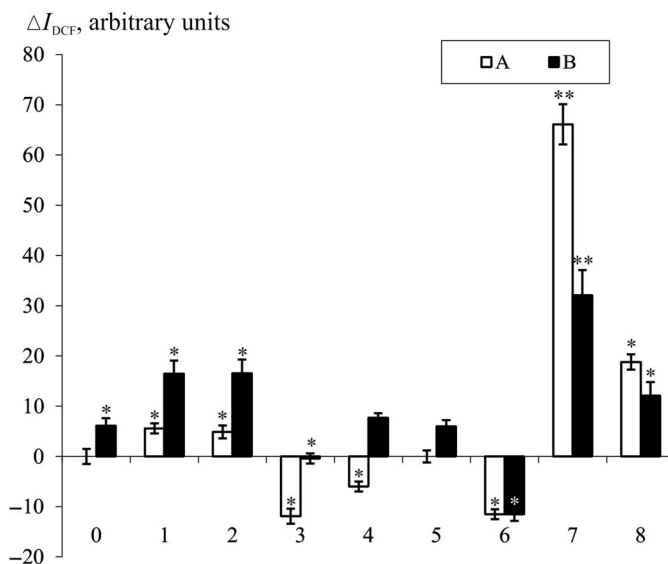
$\triangle I_{DCF}$, arbitrary units

Fig. 5. The change of mitochondrial ROS production under exposure to quinones in the absence (A) and in the presence of 1 μM rotenone (B) under Glu + Mal oxidation: 0, control; 1, BQ (1 μM); 2, DTBBQ (10 μM); 3, TMBQ (10 μM); 4, coenzyme Q_0 (10 μM); 5, coenzyme Q_{10} (10 μM); 6, menadione (10 μM); 7, juglone (1 μM); 8, lawsone (10 μM). Zero level corresponds to ROS production by intact mitochondria; *Statistically significant in comparison with the corresponding control without quinone, $p < 0.05$. **Statistically significant in comparison with the corresponding control without quinone, $p < 0.01$

Effect of quinones on the ROS generation in mitochondria
To elucidate the role of redox processes in the quinone effects on mitochondrial functions, we determined the quinone-induced ROS release to the extramitochondrial medium (Fig. 5). It was found that 10-μM DTBBQ and lawsone or 1-μM BQ or juglone increased ROS generation in rat liver mitochondria oxidizing Complex I substrate Glu+Mal. Maximal amount of ROS was registered under the action of juglone. On the contrary, a decrease of ROS production in mitochondria was revealed on TMBQ, menadione, and coenzyme Q_0 addition. Coenzyme Q_{10} did not affect ROS generation.

The inhibition of Complex I by rotenone led to the increase in Glu+Mal-stimulated ROS production in control mitochondria and in the presence of DTBBQ and BQ. However, rotenone partially inhibited juglone- and lawsone-induced ROS generation and prevented inhibition of ROS production by TMBQ and coenzyme Q_0. Complex I inhibition did not influence mitochondrial ROS production in the presence of menadione and coenzyme Q_{10}.

Effect of quinones on mitochondrial membrane potential and ROS generation in C6 cells
Figure 6 shows the alteration of mitochondrial membrane potential in glioma cell exposed to 10-μM quinones for 30 min. It is seen that in contrast to isolated mitochondria, a decrease in mitochondrial potential in intact cells was detected during treatment with all the quinones, except for coenzyme Q_{10}. It was found that juglone addition led to practically complete mitochondrial depolarization, while BQ and menadione induced 50% loss of mitochondrial membrane potential. In cells exposed to TMBQ, coenzyme Q_0, and lawsone, the mitochondrial membrane potential was 20%–30% less than in the control cells. At the same time,
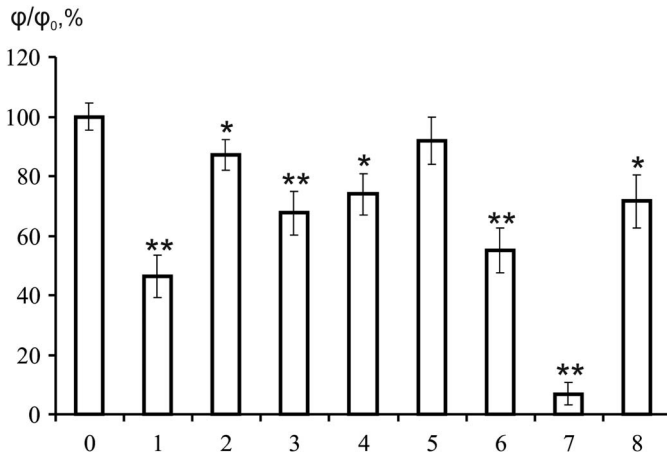
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φ/φ₀,%

Fig. 6. Mitochondrial membrane potential of C6 glioma cell exposed to 10 μM quinones for 30 min: 0, control; 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone. Each value of mitochondrial membrane potential has been divided on corresponding value φ_0 of the potential in control samples and represented as a percentage. Zero level corresponds to uncoupling at FCCP addition. *Statistically significant in comparison with the corresponding control without quinone, $p < 0.05$. **Statistically significant in comparison with the corresponding control without quinone, $p < 0.01$

DTBBQ changed the potential weakly: the mitochondrial membrane potential in treated cells was (87 ± 5)% relative to control.

Figure 7 demonstrates the mitochondrial superoxide generation levels in C6 cells exposed to 10 μM quinones. It was shown that, similarly to isolated mitochondria, BQ, DTBBQ, juglone, and lawsone induced additional $O_2^{\bullet-}$ production, while TMBQ, coenzyme
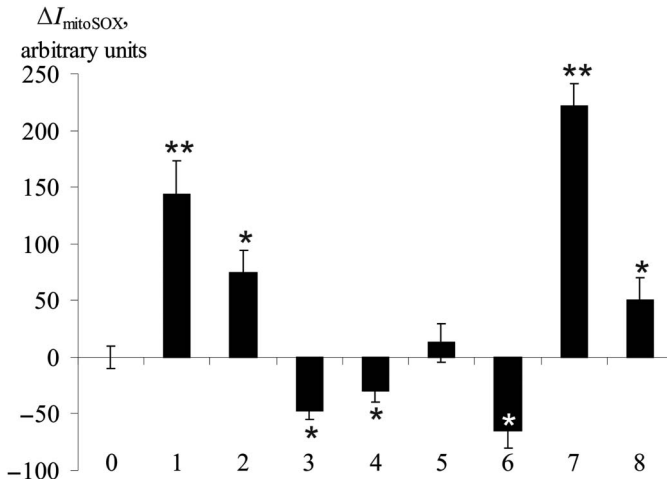
$\Delta I_{\text{mitoSOX}}$, arbitrary units

Fig. 7. The change of mitochondrial superoxide anion radicals production in C6 glioma cells under exposure to 10 μM quinones: 0, control; 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone. Zero level corresponds to mitochondrial superoxide production by intact cells. *Statistically significant in comparison with the corresponding control without quinone, $p < 0.05$. **Statistically significant in comparison with the corresponding control without quinone, $p < 0.01$

Q_0, and menadione decreased $O_2^{\bullet-}$ yield in mitochondria of intact cells. However, it is noteworthy that coenzyme Q_0, menadione, and juglone induced significant cytosolic ROS production in cells (data not shown).

Discussion

It is well known that quinones considerably influence mitochondrial function ([5], [20], [21], [26], [51]). Reduction of quinone by mitochondrial ETC complexes or DT-diaphorase can result in formation of semi- or dihydro- forms of quinone molecules. Electrochemical behavior of semi- or dihydroquinones (quinoles) can be different ([6], [38]). Reduced quinones can autoxidize, inducing ROS generation, or can transfer electrons to Complexes II, III, or IV, shunting ETC functions, changing redox balance in mitochondria, and working as potent antioxidants ([13], [23], [37], [46], [47], [49], [55]).

In our experiments, exogenous quinones considerably changed mitochondrial respiration, membrane potential, and ROS generation for isolated mitochondria or whole cells. Enhanced oxygen consumption by mitochondria (the increased V_1 and V_2 rates), which we registered in the presence of some quinones, can be due to both activation of substrate oxidation and ROS production in the quinone redox cycle with O_2. Earlier, Briere et al. ([5]) suggested that either idebenone or decylubiquinone enhanced succinate-dependent oxygen uptake in mitochondria due to significant stimulation of an electron flow from succinate to oxygen and activation of succinate dehydrogenase. On the other hand, Kelso et al. ([28]) showed that quinone derivative mitoQ significantly stimulated coupled glutamate/malate-dependent respiration of mitochondria by increasing a proton leak through inner membrane. The authors concluded that mitoQ was reduced and oxidized (recycled) by the mitochondrial respiratory complexes ([28]).

Depending on the structure and properties, the quinones affected mitochondrial ETC functions in different ways: the more lipophilic quinones studied, juglone, menadione, and DTBBQ, with $E_{1/2}$ in the range from -99 to -260 mV, disturbed mitochondrial ETC functions, decreasing the RCR and ACR coefficients (uncoupling effect); menadione, TMBQ, coenzyme Q_0 decreased ROS generation; menadione, TMBQ restored the ETC functions during inhibition of Complex I; juglone, lawsone, DTBBQ, and BQ induced ROS generation; lawsone and coenzyme Q_{10} enhanced the RCR and ACR values; and juglone and DTBBQ catalyzed the reverse electron flow from Complex II to Complex I.

We evaluated the relationships between the solubility of the quinones (Table I) and their effects on mitochondrial ETC functions (Fig. 8). A correlation analysis was performed, disregarding coenzyme Q_{10} which did not affect mitochondrial function. This may be because coenzyme Q_{10} is a native component of the ETC and the endogenous coenzyme Q_{10} has already been in excess. From the other hand, it is known that exogenous quinones can be reduced both at physiologic sites of Complex I ETC and non-physiologic ones, which are accessible for hydrophilic compounds ([31]). Due to the high hydrophobicity (the $\log P$ for coenzyme Q_{10} is almost an order of magnitude higher than the $\log P$ for the other quinones), coenzyme Q_{10} can be beyond the solubility range in which the correlation is significant. A correlation between the ACR and RCR values (Table III) and the quinone lipophilic properties [$\log P$, the partition coefficient in octanol/water system for the quinones studied, ([53])] was found. The non-parametric Spearman's rank correlation coefficients ρ_{ACR} and ρ_{RCR} were equal to -0.49 ($P_{ACR} = 0.045$) and -0.49 ($P_{RCR} = 0.049$), respectively. However, the correlation between the ADP/O ($P_{ADP/O} = 0.43$) and the quinone lipophilic properties was not ascertained (Fig. 8). It
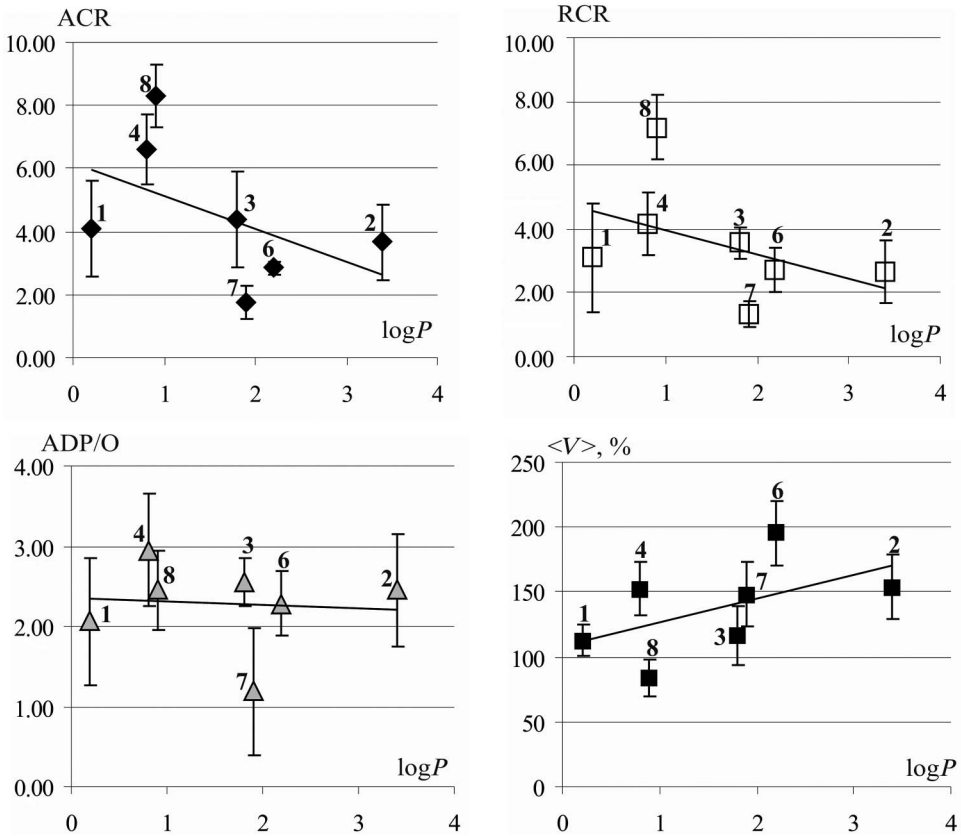
Fig. 8. Relationships between ACR, RCR, ADP/O, and $<V>$ and quinone lipophilicity: 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone

was shown previously that less hydrophobic quinone derivatives poorly affected the electron flow from either NADH or succinate to oxygen in mitochondria (5).

It is known that exogenous quinones can both be oxidized and reduced by ETC complexes (11, 13, 23, 28, 37, 46, 47, 49, 55). However, because they are not a primary substrate of these enzymes, the efficiency of oxidation/reduction has to be strongly dependent on the availability of primary substrates and "saturation" of electrons in the ETC (reduction states of components of ETC), just as the electron leak from ETC and superoxide production depend on the reduction states of ETC components and the proton gradient (25). In the absence of ADP, the mitochondrial membrane potential, stored as a proton gradient, is not consumed on ATP production and ETC is saturated, providing favorable conditions for reduction of exogenous quinones by ETC complexes, and, therefore, the greatest changes in oxygen consumption due to the reduction of quinones should be revealed for V_2 respiration rate. On the other hand, the oxidation of quinols will proceed most efficiently under conditions of lower ETC activity, namely, in the absence of respiratory substrates. The basal respiration rate V_1 is supported by a minimum of substrates that were preserved in mitochondria during isolation process. The greatest changes in oxygen consumption due to the oxidation of quinols should be registered for V_1 respiration rate. The efficiency of quinone
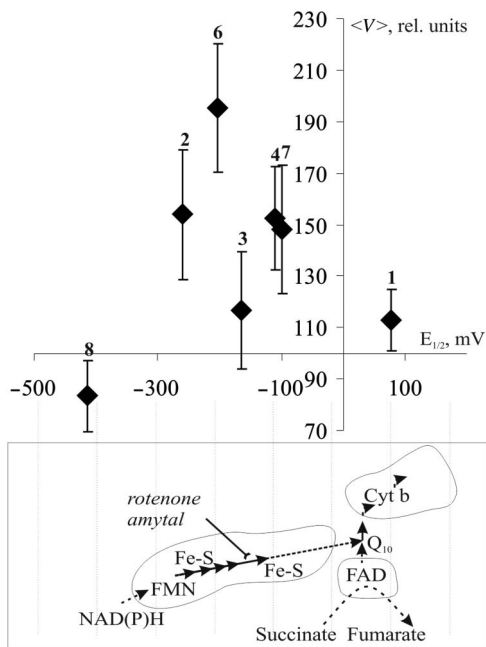
Fig. 9. Scheme for mitochondrial electron transfer with redox potential markers and relationship between $<V>$ (efficiency of quinone–ETC interaction) and quinone one-electron reduction potential: 1, BQ; 2, DTBBQ; 3, TMBQ; 4, coenzyme Q_0; 5, coenzyme Q_{10}; 6, menadione; 7, juglone; 8, lawsone

interaction with ETC depended on the processes of oxidation and reduction of quinones by ETC. Because of this, the total parameter $(V_1 + V_2)$ has to be analyzed, taking both oxidation and reduction of quinones into consideration. Hence, $<V>$ in the presence of quinones reflects their redox activity in the ETC.

We evaluated the relationships between quinone solubility and $<V>$. A correlation between $<V>$ and log$P$ was found with the correlation coefficients ρ_V being equal to 0.44 ($P_V = 0.05$). Thus, the efficiency of quinone–ETC interaction increases and the respiration parameters (ACR and RCR) decrease with the rise of quinone lipophilicity in the range of log$P$ from 0.2 to 3.4. Figure 9 shows the dependence of $<V>$ on the quinone one-electron redox potential and schematically represents the ETC, with the elements being located according to the reduction potential values. It is seen that the $<V>$ increased and, respectively, the efficient quinone-ETC interfering occurred at the quinone redox potential values from −260 to −99 mV. The potential values from −320 mV (for NADH) to −36 mV (for ubiquinone) correspond to Complex I, whereas the $E_{1/2}$ of DTBBQ ($E_{1/2} = -260$ mV) corresponds to the potential of the NADH: ubiquinone reductase initial site, while the $E_{1/2}$ of juglone ($E_{1/2} = -99$ mV) is close to the Q-reduction site of Complex I. It is known that rotenone interrupts electron transfer in Complex I, which causes a decrease of ROS generation by Complex III and an increase of ROS generation by Complex I (3, 12, 30, 32, 33, 48, 54). Then, the ROS production by quinones, which are reduced in ETC before rotenone binding site of Complex I, would increase after Complex I inhibition and, *vice versa*, the ROS production by quinones, which are reduced at the Complex I ubiquinone-binding site that is located higher than the rotenone-binding site in ETC would be suppressed during rotenone treatment. Since DTBBQ-induced ROS generation increased while juglone-induced ROS generation decreased during Complex I inhibition (Fig. 5), we have concluded that the most probable site of electron leak to DTBBQ is the Complex I flavin site, while sites

of electron leak to juglone were likely to be localized at or close to the Q-reduction site of Complex I.

To explain the influence of rotenone (inhibitor of the Complex I) on the membrane potential of mitochondria energized by succinate (substrate of the Complex II) in the presence of juglone or DTBBQ, one can suggest that these quinones catalyze the reverse electron flow from Complex II to Complex I (rotenone completely inhibited succinate-generated potential in the presence of juglone, and partially – in the presence of DTBBQ). We assume that the reduction of juglone and DTBBQ by Complex I can alter the electron path between the ETC complexes, resulting in a reverse electron flow between Complexes I and II in succinate-energized mitochondria.

The leak of electrons from the ETC to juglone or DTBBQ produced semi- and dihydroquinones. Taking into consideration, the $E_{1/2}$, semi-quinone of juglone can readily react with molecular oxygen (6, 7), resulting in mitochondrial ROS generation (Fig. 5). Due to this, juglone and DTBBQ were more effective in disturbance of the respiratory parameters of mitochondria (Tables II and III). Quinone-induced oxidative stress in mitochondria participates in a slow decrease of the mitochondrial potential as well as in uncoupling of respiration and phosphorylation (17, 42).

Although menadione ($E_{1/2} = -203$ mV), TMBQ ($E_{1/2} = -165$ mV), and coenzyme Q_0 ($E_{1/2} = -110$ mV) possess one-electron redox potential value lying in the range of Complex I potential values, the efficiencies of quinone–ETC interaction vary markedly for these quinones. On the other hand, menadione, TMBQ, and coenzyme Q_0 decreased ROS production by mitochondria. According to Fig. 9, menadione is the most effective redox-active quinone that interferes with ETC. At the same time, menadione did not disrupt mitochondrial functioning as did juglone. On the contrary, in our experiments, menadione decreased mitochondrial ROS production and did not induce membrane potential loss. In accordance with our observations Chan et al. showed that coenzyme Q_1 and menadione (5 µmol/l) considerably prevented rotenone-induced mitochondrial ROS production and rotenone cytotoxicity as well as restored mitochondrial membrane potential and ATP production in isolated hepatocytes (9). Coenzyme Q_1H_2, idebenone, and menadiol reduced by DT-diaphorase act as superoxide radicals scavengers (9, 23, 50).

In addition, ROS production is the result of quinone autoxidation and it depends on the rate of this process. The rate of menadione autoxidation following its reduction by DT-diaphorase is 50 times less compared to juglone (7). Due to this fact, menadiol, in contrast to juglone, has time to transfer electrons to Complex III before it will be oxidized by molecular oxygen. Thus, menadiol oxidation in ETC resulted in a decrease of superoxide production and restoration of ETC functions during inhibition of Complex I.

Interestingly, TMBQ ($E_{1/2} = -165$ mV) *per se* did not affect mitochondrial activities during both Glu+Mal and succinate oxidation, but similarly to menadione, it was reduced by DT-diaphorase, inhibited mitochondria ROS generation and partially restored ETC functions during inhibition of Complex I (Table V and Fig. 4). Coenzyme Q_0 is distinguished from TMBQ by change of methyl groups to methoxy ones. Meanwhile, opposite to TMBQ, mitochondria exposure to coenzyme Q_0 led to an increase in the basal respiration rate (possibly due to the electron leak from ETC to quinones), but it did not feed electrons to Complex III. It was shown (10) that coenzyme Q_1 was optimal for preventing rotenone-mediated cell death as compared to both CoQ_0 and CoQ_2. Therefore, coenzyme Q_0 can be considered as a possible electron carrier along the ETC during inhibition of Complex I

functions ([37]). Nevertheless, it is not an effective agent for restoration of mitochondrial functions possibly due to its low lipophilicity. Comparing all the quinone compounds, which can restore mitochondrial functioning under conditions of impaired mitochondrial Complex I function, notably coenzyme Q_1 ($\log P = 2.8$), duroquinone ($\log P = 2.45$, $E_{1/2} = -260$ mV), idebenone ($\log P = 4.3$), menadione ($\log P = 2.2$, $E_{1/2} = -203$ mV), and TMBQ ($\log P = 1.8$, $E_{1/2} = -165$ mV), it can be concluded that the therapeutic agents should be sufficiently lipophilic (with the $\log P$ in the range from 1.8 to 4.3) and have a one-electron reduction potential value in the range of ($-165/-260$) mV. It should be noted that the quinone electron and steric properties depended on the aromatic ring substituents, e.g., TMBQ, CoQ_0, CoQ_1, menadione, and idebenone having at least one methyl group in the quinoid ring restored mitochondrial functions, and TMBQ, CoQ_0, and menadione inhibited ROS generation in mitochondria.

BQ ($E_{1/2} = +78$ mV) did not participate in the redox cycle and it had no effect on the mitochondrial respiration parameters and potential (Table II and Fig. 3). However, BQ potentiated the action of rotenone, i.e., Complex I activity was decreased, which is in agreement with other data ([41]), and induced ROS production by mitochondria. BQ-induced ROS generation may be connected with a quinone reaction with reduced glutathione (the reaction rate constant is 2 $\mu M^{-1} s^{-1}$) ([6]). It should be noted that juglone and benzoquinone, as compared to the other quinones studied, are the most effective arylating agents. Due to this fact, juglone and benzoquinone adverse effects on mitochondrial function can result from direct arylation of some essential SH─ groups of ETC complexes.

Lawsone ($E_{1/2} = -415$ mV) increased the ACR and RCR coefficients, but did not affect the mitochondrial potential. Nevertheless, the increase of ROS production was detected under exposure to lawsone. Since lawsone did not interfere with ETC, the lawsone-induced ROS generation was probably due to lawsone reduction by DT-diaphorase followed by its autoxidation and hydrogen peroxide generation in the extramitochondrial medium.

The effects of coenzyme Q_{10} on the mitochondrial respiration parameters or the values for membrane potential were not pronounced. Probably because coenzyme Q_{10} is a native component of the ETC and it has already been in excess in the mitochondria.

On the other hand, the quinones could regulate mitochondrial permeability transition and in this way influence the mitochondrial respiration and potential. It was shown earlier by Petronilli et al. ([42]) that menadione (25 μM) stimulated mitochondrial permeability transition pore opening by shifting its gating potential, and mitochondrial SH─ groups modification completely prevented menadione-induced pore opening. Similarly, Palmeira and Wallace have shown that 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone (menadione), and 2,3-dimethoxy-1,4-naphthoquinone elicited an increase in the gating potential of permeability. Benzoquinone, on the other hand, prevented induction of the mitochondrial permeability transition by all the three redox-cycling naphthoquinones due to the electrophilic, sulfhydryl-arylating reactivity of benzoquinone ([41]).

It should be noted that in cellular systems, the mechanisms of quinone effects on the mitochondrial potential are primary mediated by cytosolic ROS generation rather than by direct action on mitochondrial components. Modification of cellular homeostasis, alteration of membrane lipid organization, and induction of oxidative stress and/or increase of Ca^{2+} intracellular level can contribute to quinone-induced mitochondrial effects on the cellular level ([20], [26]).

Conclusions

Exogenous quinones differing in chemical structure, redox potential, and lipophilic properties are capable of effective participation in mitochondrial processes, interacting with the respiratory chain complexes, and controlling electron transfer, affect respiratory parameters of mitochondria, redox homeostasis in mitochondrial compartments, dissipate mitochondrial membrane potential, influence (decrease or enhance) ROS generation, and restore electron flow during ETC inhibition by rotenone in isolated mitochondria and intact cells.

DTBBQ and juglone either are inhibitors of ETC, or they accept electrons from ETC and reduce O_2 to superoxide (or both). TMBQ and menadione can participate in ETC as electron carriers, while they are reduced by DT diaphorase or accept electrons from Complex I. While reduced, they also scavenge ROS instead of generating it.

The respiration parameters characterizing the effect of quinones on mitochondrial physiology correlated with quinone lipophilicity and depended on the redox potential and chemical structure.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

Abbreviations

ETC   = electron transport chain
FCCP   = carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
ROS   = reactive oxygen species
BQ   = 1,4-benzoquinone
DTBBQ = 2,5-di-tert-butyl-1,4-benzoquinone
TMBQ   = 2,3,5-trimethyl-1,4-benzoquinone
DCF   = 2′,7′-dichlorofluorescein

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