

Effect of the Lipophilic *o*-Naphthoquinone CG 10-248 on Rat Liver Mitochondria Structure and Function

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ABSTRACT: CG 10-248 (3,4-dihydro-2,2 dimethyl-9-chloro-2H-naphtho[1,2b]pyran-5,6-dione), a β -lapachone analogue, modified the ultrastructure of rat liver mitochondria *in vitro*, in the absence of added oxidizable substrates. The condensed mitochondrial state was replaced by the orthodox or swollen state to a significant degree. The number of modified mitochondria depended on incubation time and quinone concentration, in the 25-100 μ M range. Under the same experimental conditions, mitochondrial respiration was uncoupled as indicated by the increase in the rate of succinate oxidation by controlled mitochondria in metabolic state "4" (not in state "3"), and by the activation of latent F_0F_1 -ATP synthase. Taking into account structural similarities, the results reported here may be valid for other *o*-naphthoquinones, such as β -lapachone.

Abbreviations: CG-NQ, CG 10-248 *o*-naphthoquinone; DMFA, dimethylformamide.

Introduction

Lipophilic *o*-naphthoquinones are naturally occurring quinones that possess a variety of pharmacologic actions, as illustrated by antibacterial, antifungal, trypanocidal and cytostatic effects (Molina Portela *et al.*, 1991; Stoppani *et al.*, 2000; Dubin *et al.*, 2001). β -Lapachone (3,4-dihydro-2,2,dimethyl-2H-naphtho[1,2b]pyran-5,6-dione) exerts cytotoxic activity against many cancer cells such as Yoshida and Walker sarcoma, epidermoid laryngeal carcinoma, melanoma, promyelocytic-leukemia, lymphoma, prostate, breast, ovary, colon, hepatoma and

lung cancer cells, among other tumors (Ferreira de Santana *et al.*, 1968; D'Albuquerque *et al.*, 1972; Docampo *et al.*, 1979; Schaffner-Sabba *et al.*, 1984; Chau *et al.*, 1998; Frydman *et al.*, 1997; Wuerzberger *et al.*, 1998 and Dolan *et al.*, 1998). Moreover, β -lapachone may produce apoptosis or necrosis by complex mechanisms, not yet completely clarified (Shiah *et al.*, 1999), that involve DNA fragmentation, as well as topoisomerase, poly(ADP-ribose)polymerase, NAD(P)H-quinone reductase and caspases activities. Mitochondria play an essential role in apoptosis (Kroemer, 1999). β -Lapachone produces mitochondrial swelling in hepatoma cells and cytochrome c release in other tumor cells. The latter effect fits in well with the perturbation of mitochondrial membranes by the quinone (Planchon *et al.*, 1995; Frydman *et al.*, 1997; Vanni *et al.*, 1998; Lai *et al.*, 1998; Wuerzberger *et al.*, 1998; Planchon *et al.*, 1999). Cytochrome c release from mitochondria implies several possible mechanisms,

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namely permeability transitions or perturbation of the mitochondrial membrane. Such release plays a major role in myeloma apoptosis, specially in caspase activation, PARP cleavage and DNA fragmentation (Li *et al.*, 1993).

In order to find more potent *o*-naphthoquinones than those naturally occurring, Schaffner-Saba *et al.* (1984) synthesized a series of β -lapachone analogues with modifications at position 2 of the pyran ring or at position 9 of the naphthoquinone ring. Among these new naphthoquinones stands CG-NQ (3,4-dihydro-2,2-dimethyl-9-chloro-2H-naphtho-[1,2b]pyran-5,6-dione) that proved to be one of the more active quinones. This quinone exerted antitumor and antiviral activities, induced oxidative damage in trypanosomatids, and inhibited microsomal lipid peroxidation and cytochrome P450 catalyzed reactions (Dubin *et al.*, 1990; Molina Portela *et al.*, 1996a, b).

Taking into account the foregoing, in the present study we examined the action of CG-NQ on isolated rat liver mitochondria structure and oxidative phosphorylation activity. It is known that mitochondria may alter their internal conformation between two extreme states: the orthodox state that is usually observed in intact tissues, and the condensed state in which there is a dramatic contraction of the inner membrane accompanied by fluid accumulation in the outer compartment. The orthodox-condensed transition depends on external ADP concentration. In the condensed state the crests in the inner membrane are not visible and the matrix is more homogeneous and electron dense, whereas the outer compartment is considerably enlarged. In isolated mitochondria cycles of conformation correlate with oxidative phosphorylation (Hackenbrock, 1968; Sjöstrand, 1991). Finally, damage to mitochondrial membranes leads to swollen mitochondria in which the external membrane is disrupted, the inner membrane expanded, and the cristae disappear.

The present study demonstrates that CG-NQ produces significant alterations of rat liver mitochondria

structure, uncoupling of oxidative phosphorylation and activation of latent F_0F_1 -ATP synthase. The observed effects seem to be unrelated to quinone redox cycling and to the resulting oxygen radicals production.

Materials and Methods

Animals

Male Wistar rats (220-250 g) were used in the experiments. Animals were fed a Purina-like rat chow. The protein content of the diet was 23% and included all the essential amino acids (Dubin *et al.*, 1994).

Ultrastructure

After incubation for the time indicated under Results, mitochondria were centrifuged at 3,000 rpm. The pellet was fixed with 1.5% (w/v) glutaraldehyde dissolved in 0.2 M cacodylate buffer at pH 7.4-7.6, for 24 h at 4°C. Pellets were then centrifuged at 3,000 rpm for 15 min and washed with 0.32 M sucrose in the same cacodylate buffer, in three 15-min washes. For refixation, the same buffer solution supplemented with 1.5% (w/v) osmium tetroxide was applied for two h at 4°C. After three 15-min washes with distilled water, pellets were resuspended for 2 h in 2% (w/v) uranyl acetate and immediately dehydrated for pre-embedding. After embedding, ultrathin sections were stained with uranyl acetate in water. Microphotographs were obtained by means of a C10 Zeiss Electron Microscope using Kodak 4489 Films.

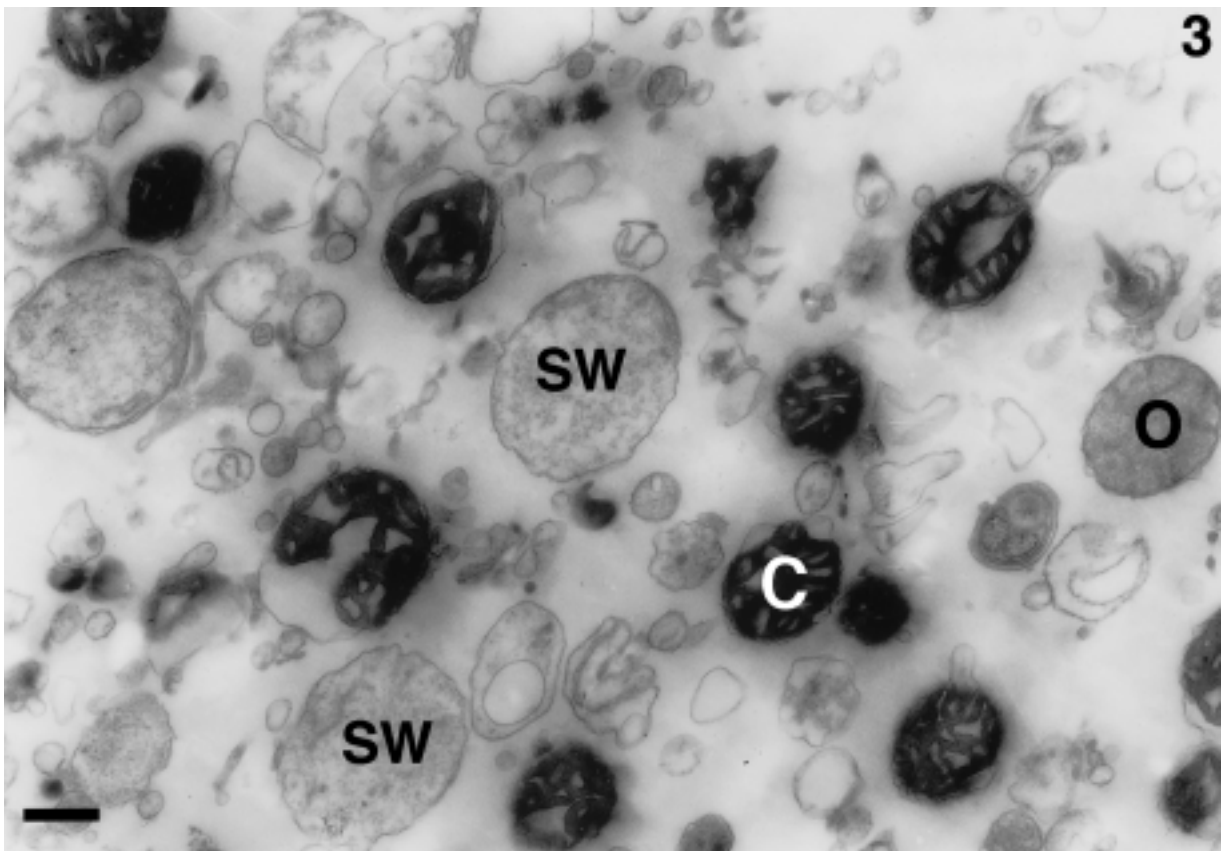
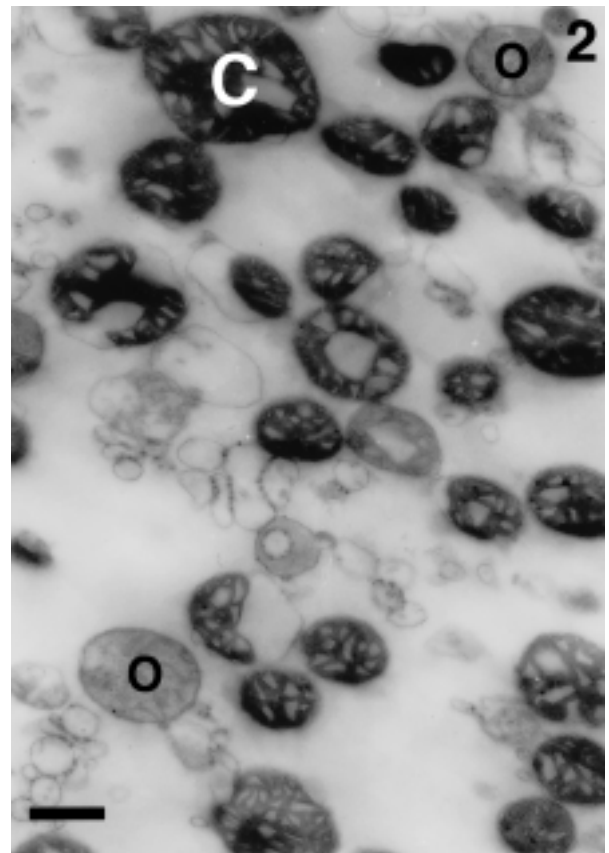
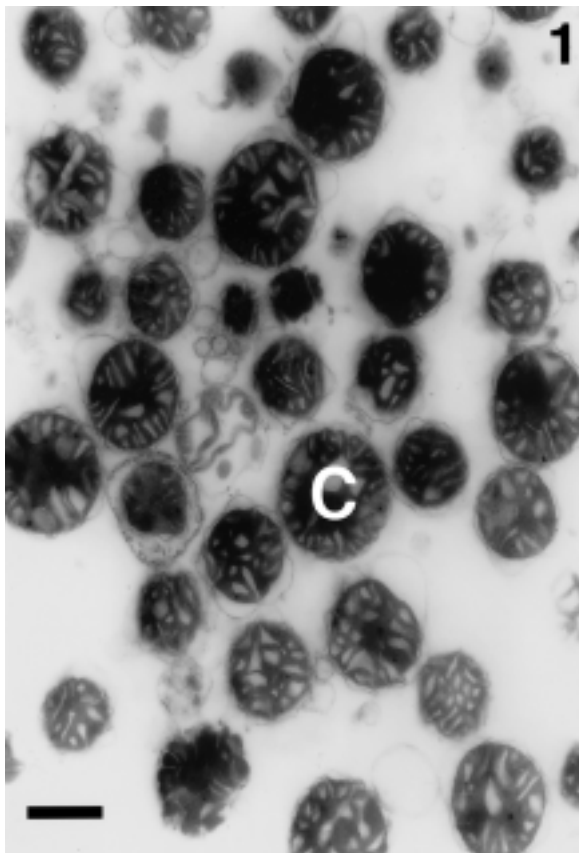
Mitochondrial preparations

Mitochondria were prepared from starved rats as described (Johnson and Lardy, 1967), using 0.24 M sucrose, 1.0 mM EDTA, 50 mM Tris-HCl buffer, pH 7.4,

FIGURE 1. Control mitochondria after 0-min incubation. Mitochondria (2.28 mg protein/ml) suspended in sucrose/EDTA/Tris-HCl medium; total volume, 1.0 ml. Mitochondria in condensed state (C). 10,000 X. Bar: 1 cm \equiv 0.6 μ m. Other experimental conditions in Materials and Methods.

FIGURE 2. DFMA-treated mitochondria, after 5 min incubation: 10 μ l DMFA/ml suspension. Mitochondria in condensed (C) or orthodox (O) state. 6,300X. Bar: 1 cm \equiv 0.4 μ m. Other experimental conditions in Materials and Methods and Figure 1 legend.

FIGURE 3. CG-NQ treated mitochondria after 5 min incubation. CG-NQ, 100 μ M. Mitochondria in condensed (C), orthodox (O) or swollen state (SW). 10,000X. Bar: 1 cm \equiv 0.4 μ m. Other experimental conditions in Materials and Methods and Figure 1 legend.



as homogenization medium. After two washes in the centrifuge at 6,800 x g with the latter buffer solution, mitochondria were suspended in the same medium at a concentration of roughly 25 mg protein/mL. When necessary, mitochondria were disrupted by freezing-thawing three times in liquid nitrogen ("uncoupled mitochondria").

Measurement of respiration rates

Respiration rates were measured polarographically, with a model 5/6 Oxygraph (Gilson Medical Electronics, Middleton, WI, USA), fitted with a Clark oxygen electrode, at 30°. For mitochondrial respiration, the stan-

dard reaction mixture (1.8 ml) contained mitochondria (1.0 to 2.0 mg protein/ml), 0.24 M sucrose, 34 mM KCl, 5.0 mM MgCl₂, 0.9 mM EDTA, 9.0 mM Tris-HCl, and 6.0 mM Na₂HPO₄-KH₂PO₄ buffer, pH 7.4. With succinate as respiratory substrate, 10 mM succinate and 3.0 µM rotenone were added. With L-malate and L-glutamate as respiratory substrates, 5.0 mM L-malate, 5.0 mM L-glutamate and 2.5 mM malonate were added. The rate of mitochondrial respiration was measured in metabolic state "3", after adding 0.5 mM ADP, or in metabolic state "4", without ADP. The respiratory control index was calculated as the ratio of state "3"/state "4" respiration (Chance, 1957).

TABLE 1.

Effect of CG-NQ on substrate oxidation by isolated rat liver mitochondria

CG-NQ (µM)	Rate of oxidation (ng atom O/min per mg protein)		
	State "3" (A)	State "4" or "3u" (B)	A/B
	<i>Substrate: 10 mM succinate</i>		
0	148 ± 16	31.3 ± 3.2	4.77 ± 0.14
2.5	152 ± 18	34.7 ± 3.9	4.46 ± 0.27
10	159 ± 17	53.6 ± 4.8*	2.98 ± 0.11
25	155 ± 19	59.7 ± 6.4*	2.63 ± 0.16
50	134 ± 14*	62.0 ± 6.9*	3.18 ± 0.07
100	122 ± 14	61.8 ± 7.0*	2.18 ± 0.07

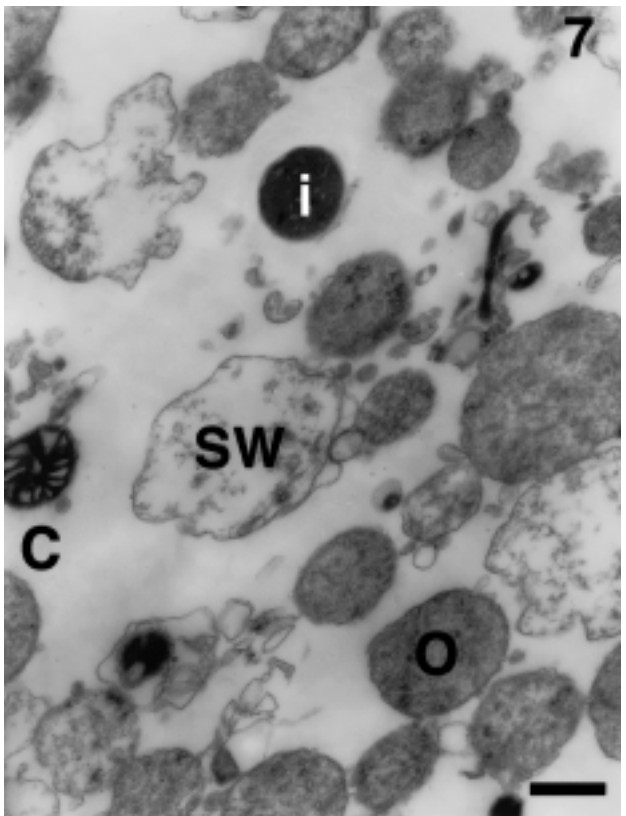
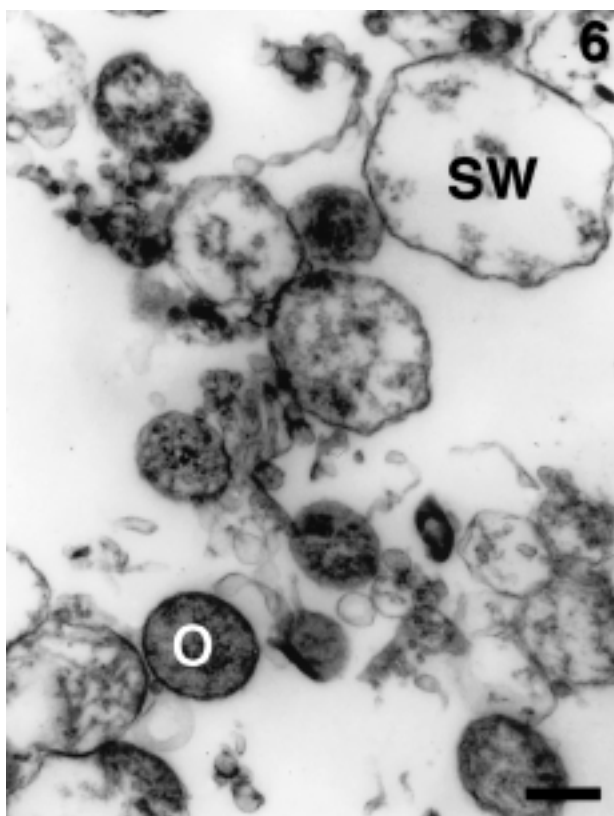
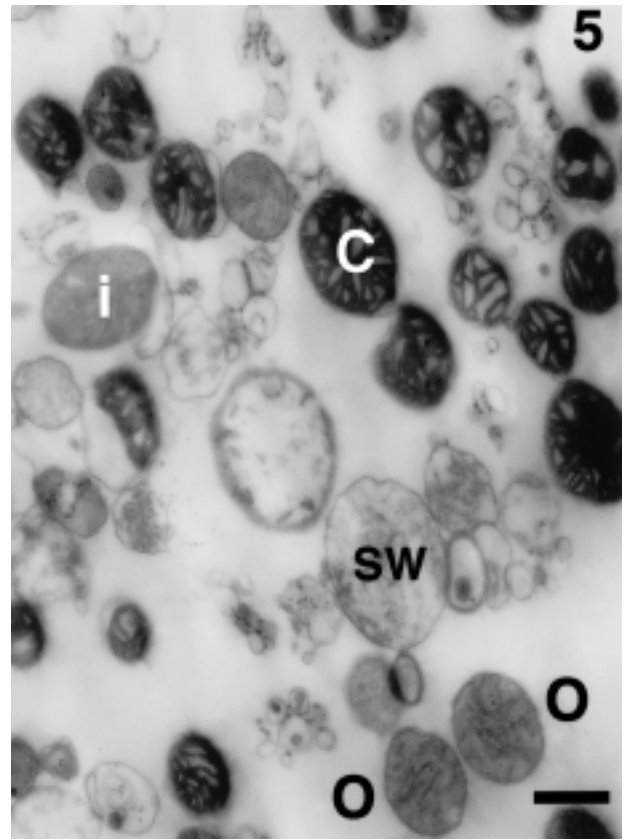
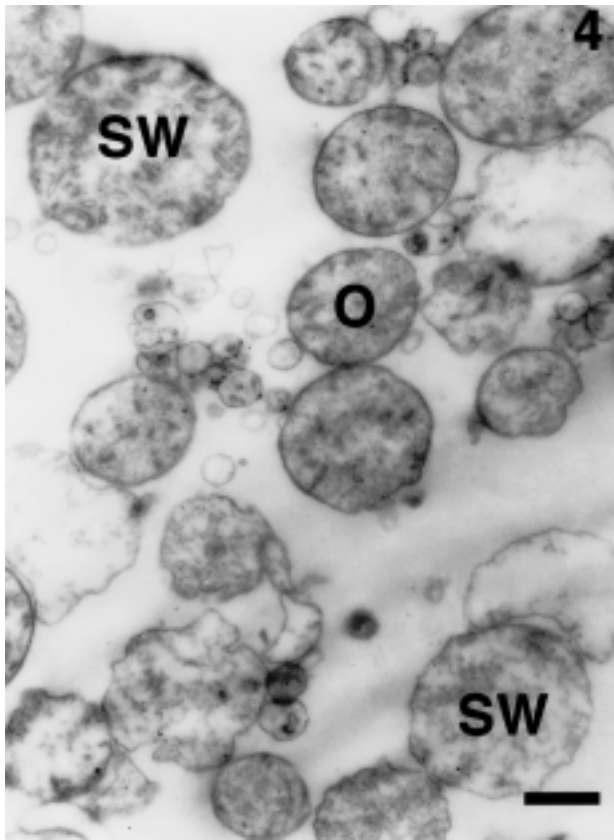
The reaction mixture contained mitochondria (1.0-2.0 mg protein/ml), 0.24 M sucrose, 34 mM KCl, 5.0 mM MgCl₂, 0.9 mM EDTA, 9.0 mM Tris-HCl and 6.0 mM KH₂PO₄-Na₂HPO₄, pH 7.4. The rate of mitochondrial respiration in metabolic state "3" was measured after addition of 0.5 mM ADP. Oxygen uptake was measured polarographically. Other experimental conditions are described under Materials and Methods. Values are means ± SD of 6 duplicate independent measurements. * P < 0.013.

FIGURE 4. DFMA-treated mitochondria, after 60 min incubation. Mitochondria in orthodox (O) or swollen (SW) state. 8,000 X. Bar: 1 cm ≡ 0.5 µm. Other experimental conditions in Materials and Methods and Figure 1 legend.

FIGURE 5. DFMA plus ADP-treated mitochondria, after 60 min incubation, ADP, 0.2 mM. Mitochondria in condensed (C), intermediate (I), orthodox (O) or swollen (SW) state. 6,500 X. Bar: 1 cm ≡ 0.6 µm. Other experimental conditions in Materials and Methods and Figure 1 legend.

FIGURE 6. CG-NQ treated mitochondria, after 60 min incubation. CG-NQ, 100 µM. Mitochondria in orthodox (O) or swollen (SW) state. 6,300 X. Bar: 1 cm ≡ 0.6 µm. Other experimental conditions in Materials and Methods and Figure 1 legend.

FIGURE 7. CG NQ plus ADP-treated mitochondria, after 60 min incubation. CG-NQ, 100 µM; ADP, 0.2 mM. Mitochondria in condensed (C), intermediate (I), orthodox (O) or swollen (SW) state. 10,000 X. Bar: 1 cm ≡ 0.6 µm. Other experimental conditions in Materials and Methods and Figure 1 legend.



Assay of F_0F_1 -ATP synthase activity

ATPase activity was measured at 30°C in an incubation mixture (final volume, 1.0 ml) containing 0.15 M Tris-HCl (pH 7.6), 3.0 mM ATP, 4.0 mM MgCl₂ and 0.25 mM EGTA. The reaction was started by adding the mitochondria. After 20 min incubation, 0.1 ml of 50% (w/v) trichloroacetic acid was added. Orthophosphate concentration was determined by the method of Fiske and Subbarow (1925).

Protein concentration

This was measured by the method of Gornall *et al.* (1949).

Material

CB NQ was kindly supplied by Novartis (formerly CIBA-Geigy), Basle, Switzerland. It was added to assay samples dissolved in DMFA (10 µl/ml mitochondrial suspension). ADP, ATP, L-malate, L-glutamate, L-malonate, succinate, rotenone, sucrose and other analytical grade reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA. Poly/Bed® 812 Resin was purchased from Polysciences Inc, Warrington, PA 18976, USA. Other reagents were analytical grades, as used previously (Dubin *et al.*, 1990).

Expression of results

When values presented were the mean of duplicate measurements, experimental values deviated from the mean by less than 5%. When more than two samples were measured, values are presented as means ± SD. Statistical analysis was performed using Student's t-test for paired samples. Unless stated otherwise, the effect of CG-NQ was calculated by taking as the control value the activity in the DMFA-containing sample, at the same incubation time and DMFA concentration (10 µl DMFA/ml mitochondrial suspension).

Results

Effect of CG-NQ on mitochondrial structure

Figures 1 and 2 show the structure of isolated mitochondria incubated without specific additions or with DMFA (control, mitochondrial samples). It is to be seen that about 95% of control mitochondria were in the condensed state and 5% in the orthodox state. Figure 3 shows the structure of mitochondria incubated for 5 min with 100 µM CG-NQ. Under these conditions, 46% mitochondria were in the condensed state, 17% in the orthodox state and the remainder were swollen. Figure 4 shows the structure of control mitochondria incubated

TABLE 2.

Effect of CG-NQ on ATP hydrolysis by coupled and uncoupled mitochondria

CG-NQ (µM)	ATPase activity (m-units/mgprotein)	
	Coupled mitochondria	Uncoupled mitochondria
None	32 ± 9	186 ± 40
5	30 ± 7	173 ± 50
10	32 ± 8	174 ± 50
25	39 ± 7	180 ± 40
50	53 ± 6*	187 ± 60
100	58 ± 8*	173 ± 50

ATPase activity was measured as described under Materials and Methods. The reaction mixture contained 0.32-0.68 mg protein/ml. Coupled mitochondrial samples were immediately used after preparation. Uncoupled mitochondrial samples were subjected to three cycles of freezing-thawing. Values represent means ± SD of at least 4 independent measurements.

* P < 0.004. Other differences, not significant.

for 60 min with DMFA, without quinone. Mitochondria were 55% in the orthodox state and 45% in the swollen state; no condensed mitochondria were observed. Figure 5 shows that addition of ADP prevented mitochondrial alterations in control samples to a significant extent, as shown by the following distribution values: 74% in the condensed, 15% in the orthodox, 7% in the swollen and 4% in the intermediate state. Incubation of mitochondria with 100 μM CG-NQ for the same time (Fig. 6) produced the greatest observed alterations: 70% were in the swollen and 30% in the orthodox state; again, no condensed mitochondria were observed. Finally, addition of ADP counteracted the effect of CG-NQ to a significant degree since the conformation-distribution values were 8% (condensed state); 47% (orthodox state) and 45% (swollen state) (Fig. 7). Comparison of CG-NQ effects under the above described experimental conditions demonstrates that mitochondrial alterations depended on (a) the time of incubation by comparing 0 min/5 min/60 min incubation samples, and (b) the presence of ADP which substantially controlled mitochondrial respiration (Table 1). A third factor affecting the mitochondrial structure modification was CG-NQ concentration. This latter effect is clearly represented in Figure 8 which shows that in the absence of quinone or at quinone concentrations within the 0-25 μM range, the orthodox/swollen mitochondria ratio was greater than > 1.0 whereas at quinone concentra-

tions within the 50-100 μM range the orthodox/swollen ratio was less than 1.0. The same occurred in the presence of ADP (experimental data omitted).

Effect of CG-NQ on mitochondrial respiration

Table 1 shows the effect of increasing CG-NQ concentrations on coupled mitochondrial respiration, using succinate as respiratory substrate. CG-NQ was used in the same concentration range employed for mitochondrial structure modification. Presented values show that (a) with mitochondria supplemented with ADP (state "3") the quinone failed to affect (low quinone concentrations) or inhibited (high quinone concentrations) the rate of respiration; (b) with the same mitochondria in the absence of ADP (state "4"), CG-NQ increased the rate of respiration about two-fold. As a result of the different effects of CG-NQ according to experimental conditions, the state "3"/state "4" ratio significantly decreased in a concentration dependent manner (Table 1). Similar results were obtained with the L-malate-L-glutamate substrate mixture (experimental data omitted).

Effect of CG-NQ on mitochondrial F_0F_1 -ATP synthase

Results in Table 2 show that, with coupled mitochondria, CG-NQ increased the rate of ATP hydrolysis as a function of naphthoquinone concentration. On the

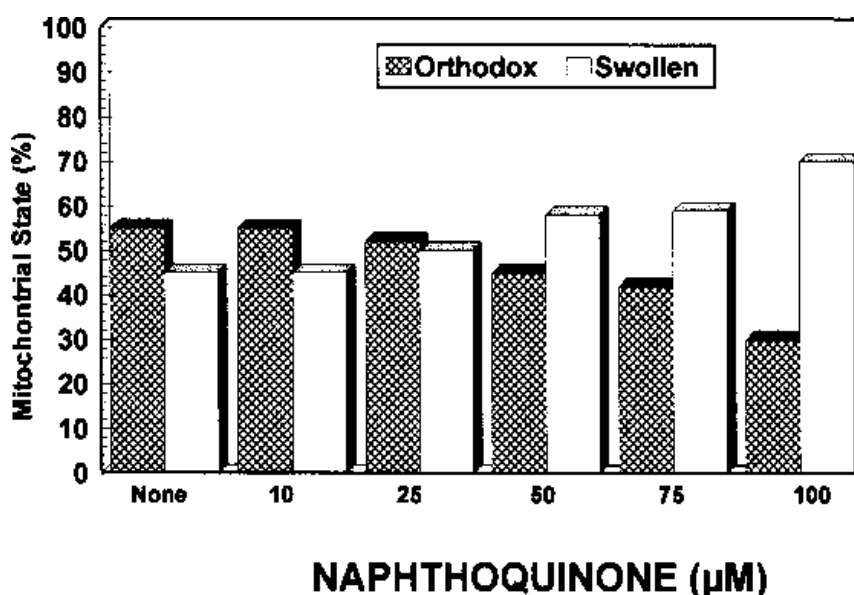


FIGURE 8. Effect of increasing CG-NQ concentration on mitochondrial conformation. Experimental conditions as in Figures 1-7, in the absence of ADP. CG-NQ concentration as stated in the abscissa. Other conditions as in Figures 1-7 legends.

other hand, uncoupling of mitochondria by repeated freezing-thawing cycles, significantly increased the rate of ATP hydrolysis that was not affected by the addition of CG-NQ.

Discussion

The observations here described demonstrate that the lipophilic *o*-naphthoquinone CG-NQ is able to modify the ultrastructure and functions of isolated rat liver mitochondria, in the absence of exogenous substrates capable of promoting quinone redox-cycling and reactive oxygen species production. The effect of CG-NQ on mitochondrial structure was clearly evidenced by the increase of mitochondria in the orthodox state and the corresponding decrease in the number of mitochondria in the condensed state (Figs. 3, 6 and 7). Moreover, CG-NQ increased mitochondrial swelling, thus producing an abnormal structural state resulting from dysfunction of ionic transport systems at the inner membrane that affected the membrane architecture and the intermembrane space (Scheffler, 2001). This membrane plays host to a set of metabolic machines constituted by the enzymes of the electron transfer chain (complexes I-IV) and ATP synthesis (F_0F_1 -ATP synthase). Complexes I, III and IV serve as proton pumps using the energy of electron transfer to pump protons from the mitochondrial matrix to the cytosol. The F_0F_1 -ATP synthase (complex V) uses proton gradient energy to synthesize ATP from ADP and P_i . The enzyme contains a membrane bound subcomplex (F_0), a large extramembrane F_1 component that resides in the matrix space and a stalk connecting F_0 and F_1 (Schultz and Chan, 2001). ATP hydrolysis (and synthesis) occurs in F_1 and is subjected to control by F_0 through the stalk. Reciprocally, binding of ADP to F_1 determines conformational

changes in the F_0F_1 -complex (Schultz and Chan, 2001) that would affect sensitivity to CG-NQ. In coupled F_0F_1 -synthase, ATP release from the enzyme-ATP complex depends on proton translocation, a reaction strictly dependent on F_1 . Uncoupling disrupts F_1 dependence on the membrane-bound mechanism and viceversa, the electron transfer activity of complexes I, III and IV is released from the "phosphate potential" control, an effect that according to Tables 1 and 2 is produced by CG-NQ. According to those data, CG-NQ would operate as a phosphorylation uncoupling agent. CG-NQ produced liberation of the ATP synthetic mechanism from the electron transfer mechanism may explain the quinone action on mitochondrial respiration (Table 1) and ATP hydrolysis (Table 2).

CG-NQ effects occur in mitochondria in the absence of exogenous substrates. Such effects support the hypothesis of a direct action of the quinone on sensitive mechanisms, not depending on quinone redox-cycling and "reactive oxygen species" production. The observations here described fit in well with β -lapachone inhibitory actions on topoisomerases (Boothman and Pardee, 1989; Li *et al.*, 1993; Planchon *et al.*, 1995; Frydman *et al.*, 1997; Wuerzberger *et al.*, 1998), and poly(ADP-ribose)polymerase (Vanni *et al.*, 1998; Fernández Villamil *et al.*, 2001). Further studies are, however, necessary for a full understanding of *o*-naphthoquinone cytotoxicity on mitochondria.

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