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# METABOLIC CONSEQUENCES OF DIETARY 2,3-DICHLORO-1,4-NAPHTHOQUINONE (CNQ) IN THE RAT

## ALTERATION IN ANTI-OXIDANT ENZYME ACTIVITIES*

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**Abstract**—Dietary exposure of rats to a high concentration of 2,3-dichloro-1,4-naphthoquinone (CNQ) (2 g/kg diet) for 60 days altered cardiac mitochondrial function and activities of anti-oxidant enzymes in hepatic and cardiac tissue. CNQ moderately depressed the cardiac mitochondrial respiratory control ratio (RCR) to 85% of control; this was exacerbated to 60% of control in animals fed α-tocopherol-deficient diets. Dietary CNQ increased hepatic superoxide dismutase (SOD) and catalase activities and increased cardiac SOD activity, but depressed cardiac glutathione reductase and hepatic glutathione peroxidase activities. These effects are consistent with previous *in vitro* findings that CNQ induces oxidative stress. No significant differences in heart weight or body weight were observed in rats fed CNQ as compared to untreated controls.

Several studies *in vitro* indicate that the fungicide CNQ (2,3-dichloro-1,4-naphthoquinone, Dichlone) alters cellular energy metabolism by inducing oxidative stress at the organelle level. CNQ inhibits mitochondrial respiration, generates $O_2^-$ and $H_2O_2$, and induces peroxidation of mitochondrial membranes [1]. It also induces large amplitude swelling of isolated rat liver mitochondria through an energy-independent, osmotic process; this process is non-specific with respect to cations [1]. CNQ-induced mitochondrial swelling requires oxygen and can be inhibited by α-tocopherol [2] or cysteine [3]. Recently, CNQ was shown to interact directly with mitochondrial thiol groups *in vitro* [4]. These *in vitro* findings imply that CNQ induces oxidative stress by direct interaction with mitochondrial sulfhydryl groups.

In addition to mitochondrial toxicity, another effect of CNQ may be alteration in the activities of enzymes which detoxify certain reduction products of oxygen. These "anti-oxidant" enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR). It has been shown that the activities of SOD [5–8], CAT [9, 10] and GPX [11–13] increase when organisms are subjected to oxidative stress caused by redox-active compounds or by oxygen itself.

Accordingly, to assess the ability of CNQ to induce oxidative stress *in vivo* we investigated its effects on two targets: mitochondrial function and anti-oxidant enzyme activity. We report the effects of a high level of dietary CNQ on (1) mitochondrial respiration and the respiratory control ratio (RCR) in rat cardiac mitochondria and (2) SOD, CAT, GPX and GR activities in both rat cardiac and hepatic tissues. Dietary α-tocopherol levels were also manipulated to determine if α-tocopherol inhibits the effects of CNQ on mitochondria *in vivo* as it does *in vitro* [2].

### MATERIALS AND METHODS

*Animal feeding*. For the mitochondrial respiration experiments, mature female Wistar rats (200–250 g, Harlan Sprague–Dawley Co., Indianapolis, IN) were fed *ad lib.* for 60 days on a Vitamin E Test Diet-Rat (U.S. Biochemical Corp., Cleveland, OH) containing 66% glucose, 10% tocopherol-stripped corn oil, 20% vitamin-free casein and 4% salt mix plus vitamin supplements free of vitamin E [14]. This diet was supplemented with 0 or 271 I.U./kg diet α-tocopherol (Type V; Sigma Chemical Co., St. Louis, MO), combined with 0 or 2 g/kg diet CNQ (MCB reagents; MCB Manufacturing Chemists, Inc., Los Angeles, CA). Four dietary groups of eight animals each were used. The start of feeding was staggered so that half of the animals in each group were killed on the same day at the end of the 60-day feeding period.

A second group of mature female Wistar rats was used for studies of protective anti-oxidant enzymes. The preparation of experimental diets was the same as above except that three levels of α-tocopherol were tested: 0, 5 and 271 I.U./kg diet, each in the absence or presence of CNQ (2 g/kg diet). The estimated minimum α-tocopherol requirement for the rat is 5 I.U./kg diet [15]. Three animals were placed in each of the six dietary groups.

*Isolation of mitochondria and determination of the respiratory control ratio (RCR)*. For each mito-

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chondrial preparation, four rats fed the same diet were killed by cervical dislocation. The animals and their hearts were weighed as a measure of gross toxicity. The four hearts were pooled and mitochondria were prepared according to the method of Palmer *et al.* [16]. In addition to the animals fed the test diets, four untreated rats fed Purina Lab Rat Chow were killed, their hearts were pooled, and cardiac mitochondria were prepared with the same method to serve as controls.

State III (ADP-stimulated) and State IV (basal rate following ADP depletion) respiratory rates of cardiac mitochondria and RCRs (State III rate divided by State IV rate) were determined polarographically on a Gilson Oxygraph equipped with a Clark electrode by measuring oxygen concentration versus time [17]. The rates and RCRs were expressed as percentage of control values determined on the same day.

*Enzyme assays*. The livers and hearts were rapidly removed from decapitated rats and were placed separately in ice-cold phosphate buffer (0.05 M, pH 7.8, 3 ml/g wet weight of tissue). The tissues were homogenized by hand with three strokes of a Potter–Elvehjem homogenizer and were centrifuged for 10 min at 600 *g*. The supernatant fractions were decanted and stored on ice until determination of CAT [18], SOD [19], GPX [20, 21] and GR [22] activities. Proteins were determined by the method of Lowry *et al.* [23].

*Statistical analysis*. Two-way analysis of variance was used to assess the effects of dietary CNQ and $\alpha$-tocopherol on heart and body weights and on the activity of each anti-oxidant enzyme in each organ studied. The criterion of significance was $P < 0.05$.

## RESULTS AND DISCUSSION

High dose dietary CNQ (2 g/kg diet, 60 days) tended to increase the heart weight/body weight ratio in $\alpha$-tocopherol-deficient rats but not in rats fed "normal" amounts of $\alpha$-tocopherol, although this effect was not significant (Fig. 1a). This increased ratio was due to the low final body weights of the $\alpha$-tocopherol-deficient, CNQ-fed animals (Fig. 1c). These findings suggested that high dose dietary CNQ caused loss of body weight that was partially prevented by dietary $\alpha$-tocopherol. However, body weight changes over the course of the experiment in the CNQ-treated and control groups were not significantly different, although CNQ-fed animals tended to gain weight and controls lose (Fig. 1d). The heart weights of the various groups at the time of sacrifice were not significantly different (Fig. 1b). Because feeding was *ad lib.* and the amount of food and CNQ consumed was not monitored, we can not definitively correlate changes in body weight or heart weight either with CNQ or total caloric intake in individual animals.

Our preliminary results on cardiac mitochondrial respiratory rates (two replicates) suggest that dietary CNQ decreased RCRs (Table 1). In animals fed $\alpha$-

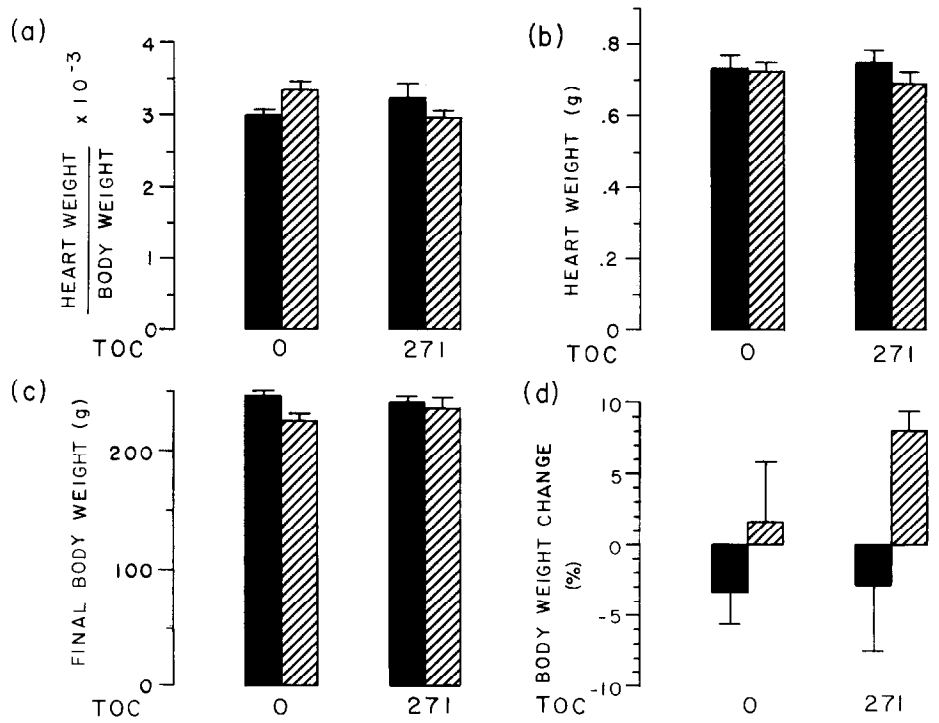


Fig. 1. Effects of dietary CNQ and $\alpha$-tocopherol on heart and body weights. In all panels, solid bars are no CNQ, hatched bars are CNQ (2.0 g/kg diet), and the numbers below the bars indicate the level of $\alpha$-tocopherol (TOC), in I.U./kg diet. Panel d: Percent change in body weight over the 60 days feeding. Values are means ± S.E.M., N = 4–8. None of the differences between groups was significant ($P > 0.05$, two-way ANOVA).

Table 1. Effects of dietary CNQ and α-tocopherol on cardiac mitochondrial RCR and State 3 and State 4 respiratory rates

| | RCR | Respiratory rate* State 3 | Respiratory rate* State 4 |
|---|---|---|---|
| α-Tocopherol 271 I.U./kg diet | | | |
| − CNQ (Chow control) | 2.5 | 32.5 | 13.0 |
| + CNQ† | 2.1 | 27.0 | 13.0 |
| (% of Control) | (84%) | (83%) | (100%) |
| No α-tocopherol‡ | | | |
| − CNQ (Control) | 2.6 | 27.8 | 10.6 |
| + CNQ† | 1.6 | 28.0 | 17.9 |
| (% of Control) | (60%) | (101%) | (169%) |
| No CNQ: | | | |
| α-Tocopherol, 271 I.U./kg diet (chow control) | 2.3 | 28.5 | 12.4 |
| α-Tocopherol-deficient | 2.1 | 31.5 | 15.0 |
| (% of Control) | (91%) | (110%) | (121%) |

All values are the average of two replicates.
* Atoms $O_2$ consumed/min/mg protein.
† Deficient diets were essentially α-tocopherol-free (see Materials and Methods).
‡ CNQ, 2 g/kg diet.

tocopherol (271 I.U./kg diet), CNQ modestly decreased the RCR to 84% of control. In contrast, the RCR was reduced by CNQ to 60% of control in α-tocopherol-deficient animals. The State 4 respiratory rate was increased to 169% of control in the CNQ-treated, α-tocopherol-deficient group. This finding suggests that CNQ causes uncoupling of cardiac mitochondria when dietary α-tocopherol is deficient. These results support our previous findings *in vitro* that CNQ causes oxidative stress in mitochondria and that α-tocopherol protects against the effects of CNQ [1–4]. In the absence of CNQ, lack of dietary α-tocopherol had little effect on cardiac mitochondrial respiration (Table 1): the RCR was slightly decreased (91% of control). Both State 3 and State 4 rates were slightly elevated in α-tocopherol-deficient animals.

High dose dietary CNQ changed the activity of several anti-oxidant enzymes in rat heart and liver (Table 2): CAT and SOD activities were increased and GR and GPX activities were decreased by CNQ. Varying the level of α-tocopherol did not affect significantly the activities of any of these enzymes (Fig. 2).

Interestingly, the pattern of change in enzyme activity was different in cardiac and hepatic tissues. Catalase activity was increased significantly only in the liver, although there was a trend toward increased CAT activity in cardiac tissue (Fig. 2a). In contrast, CNQ increased SOD activity in both heart

Table 2. Effects of dietary CNQ on enzyme activities in rat heart and liver

| Enzyme | CNQ* | Enzyme activities in tissues (units/mg protein) Heart | Liver |
|---|---|---|---|
| Catalase | − | 12.3 ± 3.9 | 50.2 ± 21.7 |
| | + | 16.7 ± 3.8 | 141.1 ± 29.0† |
| SOD | − | 82.5 ± 11.6 | 18.3 ± 6.6 |
| | + | 193.3 ± 65.0† | 56.4 ± 12.6† |
| Glutathione peroxidase | − | 34.9 ± 3.2 | 40.4 ± 8.4 |
| | + | 28.7 ± 9.0 | 19.2 ± 5.0† |
| Glutathione reductase | − | 0.024 ± 0.003 | 0.016 ± 0.005 |
| | + | 0.012 ± 0.002† | 0.018 ± 0.002 |

Values are means ± S.D., N = nine animals.
* Dietary CNQ levels: (−) indicates no CNQ, (+) indicates 2 g/kg diet. Data from groups receiving 0, 5 and 271 I.U./kg diet levels of α-tocopherol were pooled and, after two-way ANOVA, showed no significant effect of α-tocopherol ($P > 0.05$).
† The CNQ (+) group was significantly different ($P < 0.05$, two-way ANOVA) from the CNQ (−) group.

Table 3. Summary of the effects of dietary CNQ and α-tocopherol on enzyme activities in rat hearts and livers

| Enzyme | Factor | Effect* in: Heart | Liver |
|---|---|---|---|
| Catalase | CNQ | — | Increase† |
| | α-Tocopherol | — | — |
| SOD | CNQ | Increase† | Increase† |
| | α-Tocopherol | — | — |
| Glutathione reductase | CNQ | Decrease† | — |
| | α-Tocopherol | — | — |
| Glutathione peroxidase | CNQ | — | Decrease† |
| | α-Tocopherol | — | — |

All results were analyzed by two-way ANOVA (see Materials and Methods). CNQ was in the test diets at 0 or 2 g/kg diet; α-tocopherol was present at 0, 5 or 271 I.U./kg diet.
* "Increase" means that enzyme activities increased in the presence or with increasing levels of the factor; "decrease", that enzyme activities decreased.
† $P < 0.01$, two-way ANOVA. A "—" indicates differences were not significant ($P > 0.05$).

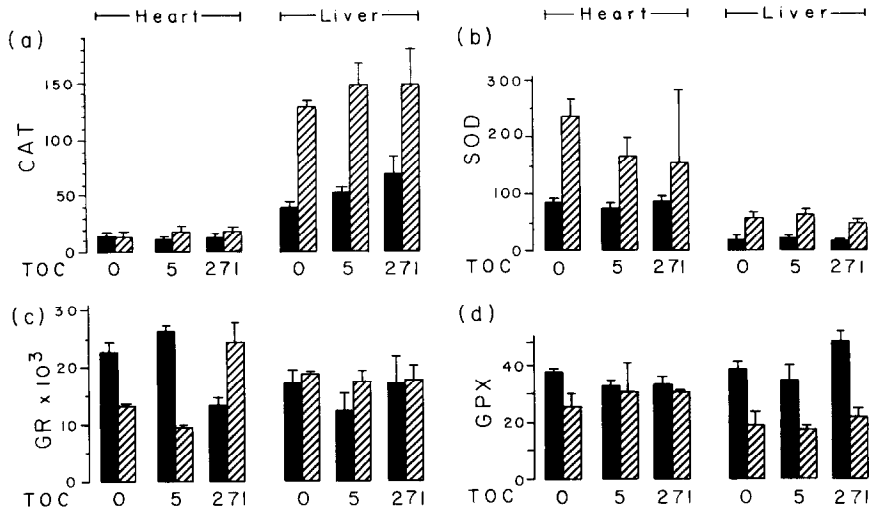


Fig. 2. Effects of dietary CNQ and α-tocopherol on the activities of anti-oxidant enzymes in heart and liver. In all panels, solid bars are no CNQ, hatched bars are CNQ (2.0 g/kg diet), and the numbers below the bars indicate the level of α-tocopherol (TOC) in I.U./kg diet. Values are means ± S.E.M., N = 3. Enzyme levels in the heart are on the left of each panel; hepatic enzymes are on the right. Enzyme activity is expressed in units/mg protein: Key: (a) catalase (CAT), (b) SOD, (c) glutathione reductase (GR), and (d) glutathione peroxidase (GPX).

and liver (Fig. 2b). GR activity was decreased by dietary CNQ only in the heart (Fig. 2c), and GPX activity was decreased significantly only in the liver (Fig. 2d). These patterns cannot be explained simply by differences in the distribution of CNQ to the two tissues because such differences would change enzyme activity in both organs proportionally to the amount of CNQ delivered to each organ. Organ-specific isoenzymes, different capacities for enzyme induction or different patterns of metabolism of CNQ in heart and liver may account for the observed patterns. The effects of CNQ and α-tocopherol on the four anti-oxidant enzymes in heart and liver are summarized in Table 3.

Our findings that CNQ increased CAT and SOD activities are consistent with others' findings that these enzymes are induced in response to oxidative stress. The toxicity of redox-active compounds such as paraquat, pyocyanine, streptonigrin, mitomycin, daunomycin, doxorubicin and porfiromycin is greater when they are administered in the presence of oxygen [5, 6, 24, 25], presumably due to enhanced formation of toxic oxygen products. Exposure of *Escherichia coli* to pyocyanine, streptonigrin and paraquat increases their SOD activity, which in turn increases their tolerance to hyperbaric oxygen [5–8]. Catalase synthesis is induced when anaerobically grown *Rhodopseudomonas spheroides* are exposed to air on $H_2O_2$ [9, 10]. CNQ, a redox active compound [4], may similarly induce these enzymes via formation of toxic oxygen products.

CNQ decreased GPX and GR activities, two enzymes thought to protect against oxidative stress. These results were unexpected in view of previous studies showing that oxidative stress induces GPX activity. For example, GPX activity increases in the intestinal mucosa and liver after oral administration of peroxidized lipids [11] and it increases in lung following exposure to 80–90% $O_2$ for 2 weeks [12] or to ozone [13]. However, CNQ may directly inhibit GPX and GR. CNQ reacts with thiol groups [4] and inhibits sulfhydryl containing enzymes [26, 27]. Since GR contains sulfhydryl groups at the active site [28], it may be a target for reaction with and inhibition by CNQ. Thus, CNQ toxicity may involve inhibition of thiol-containing enzymes in addition to production of oxidative stress. We are currently investigating these possible mechanisms.

In summary, previous experiments *in vitro* have shown that CNQ is a respiratory poison, produces $O_2^-$ and $H_2O_2$, and induces lipid peroxidation in mitochondria isolated from beef heart. CNQ undergoes redox cycling in isolated mitochondria with concomitant production of $O_2^-$ and $H_2O_2$. The results of the present *in vivo* study suggest that CNQ uncouples cardiac mitochondrial oxidative phosphorylation and induces oxidative stress in heart and liver with consequent induction of two anti-oxidant enzymes, CAT and SOD.

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