Bull. Environ. Contam. Toxicol. (1988) 41:164–171

Bulletin of Environmental Contamination and Toxicology

Modulation of Hepatic Cytochrome P-450 and DT-diaphorase by Oral and Sub-cutaneous Administration of the Pro-oxidant Fungicide Dichlone (2,3-dichloro-1,4-naphthoquinone)

Andrew J. Elliott and Ronald S. Pardini

Department of Biochemistry, University of Nevada–Reno, Reno, Nevada 89557-0014

CNQ (2,3-dichloro-1,4-naphthoquinone; Dichlone) has been applied to grain and other crops and is considered an environmental toxin (Horst, 1943; Sharvelle, 1961). It is a seed fungicide and foliage protectant and therefore can be chronically ingested by livestock and possibly other animals which eat these agricultural products.

The metabolism of CNQ has yet to be elucidated. However, according to Lind et al. (1982), quinones such as menadione (and CNQ) may undergo two paths of metabolism. The first, involving a 1-electron reduction, perhaps by cytochrome P-450 mono-oxygenases, produces a highly reactive semiquinone which in turn auto-oxidizes and generates toxic oxygen products. The second path, involving a 2-electron reduction by DT-diaphorase, produces a more stable hydroquinone which can be conjugated and eliminated. In this manner, DT-diaphorase is considered to be an antioxidant enzyme.

CNQ causes oxidative stress both in vitro and in vivo. This is evidenced by its ability to uncouple mitochondrial respiration and to cause sulfhydryl depletion in vitro (Pritsos et al., 1982; Pritsos and Pardini, 1984). Chronic CNQ ingestion in vivo increased the activity of the antioxidant enzymes catalase and superoxide dismutase (SOD), but depressed the activities of glutathione peroxidase (GP) and glutathione reductase (GR) in the rat (Pritsos et al., 1985). CNQ was further shown to be an inhibitor of GR in vitro (Pritsos et al., 1983).

Since quinone substrates have been reported to induce cytochrome P-450 enzymes (Sell, 1973; Bachur, 1979), the effect of chronic CNQ ingestion on cytochrome P-450 levels should be monitored. Induction of cytochrome P-450 could have gross effects on the organism's ability to metabolize xenobiotics since cytochrome P-450 plays an important role in detoxification and elimination of environmental chemicals. Cytochrome P-450 induction could increase susceptibility to environmental carcinogens, increase xenobiotic detoxification, and it could alter the synthesis or degradation of hormones (Anders, 1971). Furthermore, if cytochrome P-450 metabolically activates CNQ, induction of P-450 would increase the toxicity of CNQ during chronic ingestion.

Send reprint request to R.S. Pardini at the above address.

Conversely, induction of DT-diaphorase by CNQ would be expected to decrease its toxicity, if DT-diaphorase has the protective role proposed by Ernster (1974). DT-diaphorase is induced by aryl hydrocarbons which are potent inducers of cytochrome P-450 (Ernster, 1974; Hojeberg, 1981). This suggests that the two enzymes have similar structural requirements for induction.

In this study we investigated the effect of chronic ingestion, short-term (acute) ingestion, and sub-cutaneous administration of CNQ on both DT-diaphorase activity and microsomal cytochrome P-450 levels to elaborate possible mechanisms of detoxification. We also monitored a cytochrome P-450 dependent enzyme, aniline hydroxylase, as a measure of cytochrome P-450 enzyme activity following CNQ administration.

MATERIALS AND METHODS

NADH, NADPH, cytochrome C, and dicumarol were obtained from Sigma Chemical Company (St. Louis, MO). Aquasol® scintillation fluid from New England Nuclear (Boston, MA) was used. Aniline Hydrochloride[ul-14C] (S.A. 13.62 mCi/mmole) was obtained from Pathfinder Laboratories Inc. (St. Louis, MO). All other reagents used were of the highest grades available.

Isolation of cytosolic, mitochondrial, and microsomal cellular fractions was accomplished through the methods of Van der Hoeven and Coon (1974). Cytochrome P-450 content was determined spectrophotometrically from the carbon monoxide binding difference spectrum at 450-490 nm (Omura and Sato, 1978). Aniline hydroxylase activity was measured by the radiometric assay developed by Gang et al. (1972). Activity was expressed as nmoles aniline hydroxylated per minute per mg protein. DT-diaphorase activity was measured using a modified procedure of Ernster (1967). The reaction mixtures consisted of 0.4 mM NADH, 75 uM cytochrome C in 50 mM Tris buffer pH 7.6, and 20-50 ul liver extract (3.0 ml final volume). Cytochrome C reduction was monitored on an Aminco DW-2 Spectrophotometer using the dual wavelength mode at 550 minus 540 nm. Protein concentration was determined by the method of Lowry et al. (1951).

CNQ was administered in a palatable vehicle, white chocolate, to female Wistar rats (180-195 gm). Each animal was given a 2.5 gm block of the chocolate weekly in addition to food and water ad libitum. The CNQ dose groups were zero (control), medium, and high which corresponded to 0.0, 0.42, and 1.05 gm CNQ/kg-rat/week. The animals were sacrificed and their livers removed for preparation of microsomes, mitochondria and cytosol. In addition, their hearts, livers and lungs were sectioned for pathological examination. CNQ was fed orally for a period of one week or injected subcutaneously in male Long-Evans rats (180-195 gm) to estimate acute effects.

CNQ was injected subcutaneously at 16.6 mg CNQ/kg body weight into six rats. Control animals were injected with the CNQ solvent (DMSO) to determine solvent effects. At 24 and 48 hours the

animals were sacrificed and livers removed for determination of enzymatic activities. Statistical evaluation was done using the standard error of the difference of the means which was used to evaluate a significant different between two populations at $P < 0.05$ (Burns, 1972).

RESULTS AND DISCUSSION

Total cumulative CNQ intake for the chronic study was related to its concentration in the vehicle. Total intake was 0.0, 171.3 $\pm$ 63.2, and 302.8 $\pm$ 145.9 mg in the zero, medium, and high dose groups, respectively. However, weekly CNQ consumption declined to almost zero during chronic feeding for all dose groups. Animals in the two CNQ dose groups stopped eating the CNQ chocolate by the fourth week. Consumption of CNQ declined more slowly in the lower dose group and the control animals continued eating the CNQ-free vehicle throughout the study. When comparing the total CNQ intake or CNQ dose group, decrease in food consumption was not related to the concentration of CNQ in vehicle or the actual total CNQ intake (data not shown). Since the animals stopped eating the diet after four weeks of chronic feeding no change was observed for the total hepatic cytochrome P-450 content or aniline hydroxylase activity. Accordingly, no dose relationship was seen between actual CNQ intake and cytochrome P-450 content or aniline hydroxylase activity. CNQ did not appear to change the levels of dicumarol-sensitive cytosolic DT-diaphorase in the liver. In summary for chronic CNQ administration, there was no correlation between CNQ dose and cytochrome P-450 content or aniline hydroxylase activity, or DT-diaphorase activity. Food intake by and the growth rate of animals consuming CNQ were, however, transiently reduced which most likely skewed the data.

Because of the difficulty in insuring that the animals would eat the experimental diet for a prolonged period of time, we conducted a short-term experiment. For acute CNQ feeding, total cumulative CNQ intake was related to its concentration in the vehicle (Table 1).

Acute oral administration of CNQ resulted in a (n=8 per dose group, $P < 0.05$) significant increase of cytochrome P-450, DT-diaphorase, and aniline hydroxylase activities (Table 1). Aniline hydroxylase and DT-diaphorase activities directly related to the concentration of CNQ in the vehicle and actual CNQ intake. A different activation pattern at different doses of CNQ was found between cytochrome P-450 and aniline hydroxylase activities indicating that aniline hydroxylase activity may be a small and/or variable fraction of the cytochrome P-450 system. Mitochondrial DT-diaphorase activity was greater in CNQ fed than in control rats ($P < 0.05$; Table 1); thus, CNQ or a metabolite may act at the mitochondrial level as well as the cytosol.

To further investigate this CNQ-mitochondrial interaction, we found that CNQ was a substrate for both the cytosolic and mitochondrial DT-diaphorase. The activity was directly related to the orally

administered concentration of CNQ in both fractions (data not shown). From a Lineweaver-Burk plot (not shown) the apparent Km for the cytosolic DT-diaphorase was higher than that of the mitochondrial enzyme suggesting CNQ's affinity to be higher for the mitochondrial enzyme. However, the total DT-diaphorase activities with CNQ as substrate was higher in the cytosol which may also be related to CNQ compartmental movement and a cytosolic interaction. These findings are consistent with previous studies (Pritsos, 1982) which showed that CNQ inhibits mitochondrial respiration in vitro. Furthermore, evidence given here indicates that CNQ does indeed interact with the mitochondria as well as the cytosol, a finding that corroborates previous findings regarding CNQ metabolism (Pritsos, 1986). The increased DT-diaphorase activity found in the mitochondrial fraction after CNQ ingestion may result from the protective reaction of the mitochondria to prevent excessive formation of toxic oxygen species. If CNQ were metabolized by the 1-electron cytochrome P-450 scheme, a highly reactive semiquinone would be formed which in turn would auto-oxidize and form toxic oxygen species.

Animals possessed increased mitochondrial DT-diaphorase activity at 24 and 48 hours after sub-cutaneous injection with CNQ. By 48 hours post injection, all of the enzyme activities were significantly different from control values (Table 2). These findings indicate that CNQ or a CNQ metabolite also interacts first at the mitochondrial level of the cell when injected sub-cutaneously. Cytosolic DT-diaphorase was higher in the DMSO injected controls (Table 2) than in the uninjected controls (Table 1). These findings suggest that sub-cutaneous injection of DMSO increases cytosolic DT-diaphorase activities.

The results presented here suggest that the environmental toxin, CNQ, a quinone, does increase the hepatic microsomal cytochrome P-450 content, aniline hydroxylase activity, and DT-diaphorase (cytosolic and mitochondrial) activities following acute oral administration. Sub-cutaneous injection increases the mitochondrial DT-diaphorase activity at 24 and 48 hours and the cytochrome P-450 content at 48 hours. These findings indicate that the route of administration and carrier are important in determining the intracellular effects of CNQ. Previous studies (Pritsos et al., 1986; Pritsos et al., 1984) have shown that CNQ feeding increased the antioxidant enzymes, SOD and catalase of the whole heart and liver as well as liver mitochondria. These findings lead to the speculation that CNQ exposure leads to an increase in hepatic cytochrome P-450 levels and activities which, in turn, triggers the formation of a semiquinone intermediate leading to oxidative stress. In turn, hepatic levels of SOD and catalase are elevated for protection from the CNQ induced generation of toxic oxygen radicals. In addition, the DT-diaphorases of the cell are also elevated to decrease the production of semiquinone formation by competing with the cytochrome P-450 system by converting the quinone to the hydroquinone form which can then be conjugated and excreted.

Table 1. Effect of acute feeding of CNQ to female Wistar rats on hepatic enzyme activities*

CNQ Dose Group**	Actual CNQ Intake (mg ingested during course of experiment)	Cytochrome P-450 Content (nmoles/mg protein)	Aniline Hydroxylase Activity (nmoles Aniline hydroxylated /min/mg protein)
Zero CNQ (Control)	0.0	0.248 ± 0.09	0.257 ± 0.14
Med CNQ	281.1 ± 47.7	1.128 ± 0.80+	0.772 ± 0.38+
High CNQ	625.5 ± 147.4	0.962 ± 0.99+	1.231 ± 0.52+

CNQ Dose Group**	Mitochondrial DT-diaphorase Activity (nmoles reduced Cytochrome C /min/mg protein)	Cytosolic DT-diaphorase Activity (nmoles reduced Cytochrome C /min/mg protein)
Zero CNQ (Control)	0.114 ± 0.06	0.107 ± 0.08
Med CNQ	0.242 ± 0.16+	0.297 ± 0.17+
High CNQ	0.387 ± 0.26+	0.242 ± 0.20

*Each values is from an average of 8 animals ± standard deviation.

**The concentration of CNQ administered in the diet was 0, 24, and 60 mg/gm vehicle which corresponded to 0, 0.4, and 1.0 gram CNQ/kg-rat/week for the zero, medium and high groups respectively.

+Indicates that the value is significantly different from the Control value with $P < 0.05$.

Table 2. Effect of sub-cutaneously injected CNQ to male Long-Evans rats*

Group	Cytosolic DT-diaphorase Activity** (nmoles Cytochrome C reduced /min/mg protein) At 24 Hours	At 48 Hours	Mitochondrial DT-diaphorase Activity** (nmoles Cytochrome C reduced /min/mg protein) At 24 Hours	At 48 Hours
Control	1.37 ± 0.55	0.50 ± 0.15	0.228 ± 0.16	0.172 ± 0.08
CNQ	1.21 ± 0.64	1.34 ± 0.51++	0.334 ± 0.12++	0.489 ± 0.09++

Group	Aniline Hydroxylase Activity+ (nmoles Aniline hydroxylated /min/mg protein) At 24 Hours	At 48 Hours	Cytochrome P-450 Content+ (nmoles/mg protein) At 24 Hours	At 48 Hours
Control	0.141 ± 0.05	0.068 ± 0.01	0.209 ± 0.01	0.237 ± 0.01
CNQ	0.098 ± 0.04++	0.118 ± 0.01++	0.178 ± 0.05	0.393 ± 0.10++

*The amount injectected to each animal was 33.2 mg CNQ/ml of DMSO. A volume of 0.5 ml was administered to each animal.

**Each value is from an average of 18 animals ± standard deviation.

+Each value is from an average of 12 animals ± standard deviation.

++Indicates that the value is significantly different from the Control values with $P < 0.05$.

REFERENCES

Anders MW (1971) Enhancement and inhibition of drug metabolism. Ann Rev Pharmacol 11:37-56

Bachur NR, Gordon SL, Gee MV, Kon H (1979) NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc Natl Acad Sci 76(2):954-957

Burns GW (1972) The science of genetics. The Macmillin Comp, New York, New York Campbell CT, Hayes JR (1974) Role of Nutrition in the Drug-Metabolizing Enzyme System Pharmacol Rev 26(3):171

Ernster L (1967) DT-diaphorase. Methods in Enzymology 56 (Vol10): 309-317

Ernster L, Lind C (1974) A possible relationship between DT-diaphorase and the aryl hydrocarbon hydroxylase system. Biochem Biophys Res Com 56(2):392-399

Gang H, Lieber CS, Rubin E (1972) Aniline hydroxylase microassay suitable for needle biopsy specimens: Effects of phosphate and pyrophosphate on aniline hydroxylase. J Pharamcol Exp Ther 183:218

Hojeberg B, Blomberg K, Stenberg S, Lind C (1981) Biospecific Adsorption of Hepatic DT-Diaphorase on Immobilized Dicoumarol. Arch Biochem Biophys 207(1):205-216

Horst WP, Felix EL (1943) CNQ: a potent organic fungicide. Indust Eng Chem 35:1255

Lau AYH (1971) Liver microsomes and drug metabolizing enzyme system: functional components and their properties. Fed Proc 35(3):2460

Lind C, Hochstein P, Ernster L (1982) DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. Arch Biochem Biophys 276(1):178-185

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). J Biol Chem 193, 265

Omura T, Sato R (1978) Isolation of Cytochromes P-450 and P-420 Methods in Enzymology 52(Part C):556-561

Pritsos CA, Jensen JC, Pisani D, Pardini RS (1982) Involvement of Superoxide in the interaction of 2,3-dichloro-1,4-naphthoquinone with mitochondrial membranes. Arch Biochem Biophys 217:98

Pritsos CA, Pisani, DE, Pardini RS (1983) Inhibition of Glutathione Reductase and Peroxidase activity by 2,3-dichlorol,4-naptho-quinone. Fed Proc 42:2109

Pritsos CA, Pardini RS (1984) A redox-cycling mechanism of action for 2,3-dichloro-1,4-naphthoquinone with mitochondrial membranes and the role of sulfhydryl groups. Biochem Pharamacol 33:3791

Pritsos CA, Pisani DE, Pardini RS (1985) Inhibition of liver glycolysis in rats by dietary dichlone (2,3-dichloro-1,4-naph-thoquinone). Bull Environ Contam Toxicol 35:23

Pritsos CA, Aaronson LM, Pardini RS (1986) Metabolic consequences of dietary 2,3-dichloro-1,4-naphthoquinone (CNQ) in the rat Alteration in the Anti-Oxidant Enzyme Activities. Biochem Pharmacol 35:1131

Sax NI (1984) Dangerous Properties of Industrial Materials (9th ed). Van Nostrand Reinhold Co, New York, New York

Sell JL, Davison KL (1973) Changes in the activities of hepatic microsomal enzymes cause by DDT and dieldrin. Fed Proc 32(9):2003
Sharvell EG (1961) The Nature and Uses of Modern Fungicides Minneapolis. Burgess Publishing Co, MN
Van der Hoeven TA, Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. J Biol Chem 249:6302
Windholz M (ed) (1976) The Merck Index (9th ed). Merck and Co, Rahway, New Jersey

Received October 2, 1987; accepted December 16, 1987.