seen 4 days after treatment should characterize intermediate effects.

The observation that there is generally no change in the parameters measured 1 and 4 days following administration and a significant decrease after 7 days, suggests that direct alkylation by acrolein is unlikely to be the mechanism by which CP decreases mixed function oxidation *in vivo*. The suppression of cytochrome P450 was not altered when *N*-acetylcysteine was coadministered with CP providing further evidence that acrolein is not directly inactivating cytochrome P450. The decrease *in vivo* is likely to be mediated by CP itself or a CP metabolite via changes in transcription and/or translation.

It appears that CP inactivates cytochrome P450 *in vivo* and *in vitro* via different mechanisms. The differences between *in vivo* and *in vitro* results may be attributed to the pharmacokinetics of CP, CP metabolites and NAC, and protein turnover which are operative *in vivo* but absent *in vitro*. There may also be differences in the accessibility of CP, CP metabolites and NAC to cytochrome P450 and the regulatory sites of cytochrome P450 expression. Clearly, the mechanism by which CP alters cytochrome P450 in rats requires further investigation. It also remains to be determined whether such perturbations occur in humans and if so whether the outcome is of clinical significance.

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Biochemical Pharmacology, Vol. 43, No. 12, pp. 2658–2660, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

The effect of glycerol and 4-nitroquinoline 1-oxide on active oxygen formation in sub-cellular fractions of lung tissue

(*Received* 16 *January* 1992; *accepted* 31 *March* 1992)

Abstract—Glycerol enhances pulmonary tumorigenesis in mice treated with 4-nitroquinoline 1-oxide (4NQO). The present study shows that active oxygen formation by glycerol may be responsible for this enhanced tumorigenesis. Male ddY mice were treated with 4NQO and given a 5% glycerol solution instead of drinking water for up to 4 weeks after 4NQO injection. There was no difference in NADH-dependent active oxygen formation of the mitochondria between the 4NQO- and 4NQO-plus-glycerol-treated groups but the ratios of NADH- and NADPH-dependent active oxygen formation of the microsomes and nuclei of the 4NQO-plus-glycerol-treated group to the 4NQO-treated group increased with increasing time after 4NQO injection. Significant differences in the maximum NADH- and NADPH-dependent active oxygen formation were observed 2 or 4 weeks after 4NQO injection.

It has been reported that the bronchiolar non-ciliated (Clara*) cell is a possible progenitor cell of peripheral carcinoma in the lung [1]. Glycerol has been found to induce morphological changes in the Clara cell and enhance the development of lung tumors in ddY mice treated with 4NQO [2, 3]. It has been suggested that excess active oxygen species formed and lipid peroxides might augment the process of carcinogenesis ("oxidative stress hypothesis") [4–7]. Since it has been reported that marked morphological changes of the mitochondria and smooth endoplasmic reticulum in the Clara cell are caused by glycerol [2], active oxygen formation in these sites may be enhanced by such treatment. To test if increased active oxygen formation contributes to the enhancement of 4NQO-induced pulmonary tumorigenesis in mice, the effect of glycerol on active oxygen formation in the lungs of mice treated with 4NQO was measured. The previous report [3] demonstrated that the period of glycerol treatment needed for the most effective measurement of 4NQO-induced pulmonary tumorigenesis was 4 weeks after 4NQO injection. Consequently, we measured active oxygen formation at 1, 2 and 4 weeks of glycerol treatment after 4NQO injection.

Materials and Methods

The animals used were 6-week-old male mice of a specific pathogen-free ddY strain, purchased from Japan SLC (Shizuoka, Japan). The mice had free access to water and food (CE-2 chow, Clea Japan, Tokyo, Japan) throughout the study. The mice were given 4NQO solution at the dose of 10 mg/kg body weight by a single subcutaneous injection on the first experimental day [8]. Glycerol (Wako Pure Chemicals Industries, Osaka, Japan) (5%) was given instead of water *ad lib*. At 1, 2 and 4 weeks after 4NQO injection, all mice were killed by exsanguination of the abdominal artery under anesthesia with pentobarbital. A 10% lung homogenate was prepared in 1.15% KCl or 2.4 M sucrose–3.3 mM $CaCl_2$. Mitochondria, microsomes and nuclei from the homogenate were isolated by differential centrifugation [9, 10]. To monitor active oxygen formation in the mitochondria, microsomes and nuclei, lucigenin (Boehringer, Mannheim, German)-amplified chemiluminescence [11] was measured with a luminescence Reader (Aloka, Tokyo, Japan). Statistical comparison between 4NQO- and 4NQO-plus-glycerol-treated groups was performed by the use of Student's or Welch's *t*-test after analysis of variance. $P < 0.05$ was used for significant difference.

Results and Discussion

Glycerol treatment for 2 or 4 weeks after 4NQO injection did not influence active oxygen formation of the pulmonary mitochondria (data not shown). Glycerol-associated increase in microsomal NADPH- and NADH-dependent active oxygen formation was observed with increasing time (Table 1). Microsomal active oxygen formation was maximum with a significant difference 4 weeks after 4NQO injection. A time-dependent increase in nuclear NADPH- and NADH-dependent active oxygen formation occurred, and the maximum values with significant difference due to glycerol treatment were present 2 and 4 weeks after the administration of 4NQO, respectively (Table 1).

It is considered that a critical factor for 4NQO-induced tumorigenesis is the amount of ultimate carcinogen 4-hydroxyaminoquinoline 1-oxide (4HAQO)–DNA adduct in a target organ [12]. Though the enhancement of 4NQO-induced pulmonary tumorigenesis by glycerol can depend partly on increase in the 4HAQO–DNA adduct level in the lung, the effect of glycerol did not correspond to the

* Abbreviations: Clara cell, bronchiolar non-ciliated cell; 4HAQO, 4-hydroxyaminoquinoline 1-oxide; 4NQO, 4-nitroquinoline 1-oxide.

Table 1. Time-dependent alterations in NADPH- and NADH-dependent active oxygen formation in pulmonary microsomes and nuclei in response to glycerol

Substrate	Treatment period (week)	Active oxygen formation (kcounts/g lung)		
		4NQO(A)	4NQO + glycerol (B)	B/A
Microsomes				
NADPH	1	8967 ± 733	10402 ± 1233	1.16
	2	8578 ± 1792	9629 ± 1930	1.12
	4	6711 ± 666	9597 ± 805*	1.43
NADH	1	4957 ± 745	5849 ± 682	1.17
	2	5017 ± 539	6372 ± 828	1.27
	4	4767 ± 462	7294 ± 810*	1.53
Nuclei				
NADPH	1	15957 ± 4527	19787 ± 2992	1.24
	2	16273 ± 1215	25549 ± 2807*	1.57
	4	12070 ± 1906	17260 ± 917*	1.42
NADH	1	17268 ± 4527	21153 ± 2159	1.22
	2	17118 ± 3199	24606 ± 4194	1.43
	4	10210 ± 1951	16847 ± 1710*	1.65

The mixture for chemiluminescence measurement contained 0.1 M phosphate buffer (pH 7.4), 200 μM lucigenin, 200 μM NADH or NADPH and 0.2 mg of each sample protein in a final volume of 1 mL. The measurement was performed at 37° and the reaction started by the addition of NADH or NADPH.

Values for active oxygen formation are total counts of chemiluminescence per gram lung during reaction time. Each reaction time was as follows: microsomal NADH-dependent reaction, 8 min; microsomal NADPH-dependent reaction, 6 min; nuclear NADH- or NADPH-dependent reaction, 5 min.

Each value is the mean ± SEM of four determinations.

* $P < 0.05$ with respect to 4NQO-treated group.

amount of 4HAQO–DNA adduct in the lung [13]. With regard to the co-carcinogenic potential of active oxygen species and chemical carcinogens, it is possible that if DNA–chemical carcinogen adducts are present, DNA perturbations caused by oxidative stress may have an enhancing effect on carcinogenesis [14]. The maximum glycerol-associated formation of active oxygen was observed 4 weeks after 4NQO injection. Moreover, this period was in agreement with the maximum effect of glycerol on 4NQO-induced pulmonary tumorigenesis. Glycerol treatment induced the increase in active oxygen formation in the lung, thereby accounting for the promoting effect of glycerol on 4NQO-induced pulmonary tumorigenesis.

It is well established that the mitochondria, microsomes and nucleus can produce active oxygen species via NADH- and/or NADPH-dependent electron-transferring systems [5–7]. Since glycerol treatment can induce morphological changes of mitochondria in the Clara cell [2], it may stimulate active oxygen generation from the mitochondria in mice treated with 4NQO. However, there was no difference in active oxygen generation between 4NQO- and 4NQO-plus-glycerol-treated groups in this study, suggesting that superoxide dismutase and glutathione peroxidase in the mitochondria can effectively destroy active oxygen species generated in this organelle as reported previously [15]. Therefore, even if the level of active oxygen formed from the mitochondria due to glycerol treatment increased, it is possible that the capacity to eradicate this active oxygen is superior to the capacity to generate it, thus accounting for the lack of difference in active oxygen generation from mitochondria between the 4NQO-treated and 4NQO-plus-glycerol-treated groups.

Cytochrome P450 reductase and/or cytochrome P450 play an important role in NADH- and NADPH-dependent active oxygen formation in the microsomes [6]. An earlier morphometric study demonstrated that a time-dependent increase in the profile area of the smooth endoplasmic reticulum was observed in a glycerol-treated group [2], and this increase could be associated with the promotion of active oxygen generation from the smooth endoplasmic reticulum via cytochrome P450 systems. Thus, this promotion may be reflected in the time-dependent increase of active oxygen formation. The reason why active oxygen formation in the nucleus increased with increasing time due to glycerol treatment is still unclear. Since the nuclear membrane has NADPH- and NADH-dependent electron-transferring systems like the microsomes and mitochondria [7], glycerol treatment may stimulate these systems and increase active oxygen formation in the nucleus.

In summary, our present data indicate that the glycerol-related increase in active oxygen formation in the lung can partly account for the enhancing effect of glycerol on pulmonary tumorigenesis in mice treated with 4NQO.

Acknowledgement—This work was supported in part by a grant from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control.

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