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Genotoxicity of 1,4-Benzoquinone and 1,4-Naphthoquinone in Relation to Effects on Glutathione and NAD(P)H Levels in V79 Cells

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1,4-Benzoquinone is cytotoxic in V79 Chinese hamster cells and induces gene mutations and micronuclei. The cell-damaging effects of quinones are usually attributed to thiol depletion, oxidation of NAD(P)H, and redox-cycling involving the formation of semiquinone radicals and reactive oxygen species. To elucidate the role of these mechanisms in the genotoxicity of 1,4-benzoquinone, we measured various genotoxic effects, cytotoxicity, and the levels of glutathione, NADPH, NADH, and their oxidized forms all in the same experiment. 1,4-Naphthoquinone, which does not induce gene mutations in V79 cells, was investigated for comparative reasons. The quinones had a similar effect on the levels of cofactors. Total glutathione was depleted, but levels of oxidized glutathione were slightly increased. The levels of NADPH and NADH were reduced at high concentrations of the quinones with a simultaneous increase in the levels of $NADP^+$ and NAD^+. Both compounds induced micronuclei, but neither increased the frequency of sister chromatid exchange. Only 1,4-benzoquinone induced gene mutations. This effect was observed at low concentrations, where none of the other parameters studied was affected. When the cells were depleted of glutathione prior to treatment with the quinones, the induction of gene mutations and micronuclei remained virtually unchanged. We conclude that a) induction of micronuclei and glutathione depletion by the two quinones are not linked causally, b) 1,4-benzoquinone induces gene mutations by a mechanism different from oxidative stress and glutathione depletion, and c) glutathione does not fully protect the cells against the genotoxicity of quinones.

Introduction

1,4-Benzoquinone, a metabolite of benzene, is a potent mutagen in V79 Chinese hamster cells (*1*). The reason for this effect is not known, but it may involve one or several of the following mechanisms. Quinones are electrophiles and may undergo addition reactions with nucleophiles (Michael reaction). Indeed, when 1,4-benzoquinone was incubated in buffer with deoxyguanosine, adducts were formed (*2*). Alternative target structures are thiol groups and other nucleophilic moieties in proteins. Quinones may be metabolized to semiquinone free radicals, e.g., by one-electron reduction or by two-electron reduction and subsequent one-electron oxidation (*3*). The resulting semiquinone radicals are chemically reactive and may react directly with the target structure in the cell. Alternatively, they may transfer their unpaired electron onto other molecules, e.g., onto molecular oxygen leading to superoxide, or onto superoxide leading to hydrogen peroxide. Superoxide and hydrogen peroxide are not stable in biological systems and may give rise to the formation of additional reactive forms of oxygen. Superoxide, for example, may disproportionate to hydrogen peroxide singlet oxygen. Mutagenic activity was observed with hydrogen peroxide, added at high concentrations ($\geq$ 1 mM) to V79 cells (*4*). In this context, it is important to know that one molecule of quinone may give rise to the formation of numerous molecules of active oxygen, since transfer of the electron from semiquinone to another molecule results in reformation of the quinone, which is now available for new redox cycles with radical formation.

The aforementioned active species are dealt with by a number of detoxification systems. Thiols, such as glutathione (GSH), which is present in cells in large concentrations, are excellent reactants for the Michael addition. Moreover, the velocity of the conjugation of GSH with quinones may be strongly enhanced by GSH transferases (*5*). GSH, in the presence of GSH peroxidase, is also a principal system for the detoxification of hydrogen peroxide, the products being water and glutathione disulfide (GSSG) (*6*). Treatment of cells with quinones can lead to the exhaustion of the cellular GSH pool (*7,8*). This exhaustion is not only critical because the toxic species are then no longer detoxified by this system, but also be-

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cause GSH is important in various additional physiological processes, including Ca^{2+} homeostasis (*7*).

Loss of GSH through conjugation is usually irreversible. On the other side, GSH may be regenerated from GSSG by GSH reductase, with concomitant oxidation of NADPH. NADPH and NADH, depending on the reductases involved, are also oxidized in the redox cycling of the quinones. The levels and redox states of the pyridine cofactors therefore are additional cellular systems which may be disturbed by quinones (*9*).

From these reflections, possible mechanisms for the toxicological effects of quinones may be classified into three groups, depending on whether they result from a) direct reaction of the quinone or a metabolite with the cellular target structure; b) reaction of a secondary reactive species, e.g., derived from oxygen, with the target; and c) dysfunction of cellular processes owing to disturbances in cofactor pools. Even if mechanisms a and b held, it is possible that an effect becomes significant only after depletion of GSH, the cofactor used in the detoxification. We therefore studied cofactor levels, toxicity, and mutations in 1,4-benzoquinone-treated cells as functions of the exposure concentration. In comparison, 1,4-naphthoquinone, which does not induce gene mutations in V79 cells, was investigated. In addition, the mutagenicity and toxicity of 1,4-benzoquinone were studied in cells that were previously GSH depleted.

Materials and Methods

Chemicals, Cell Culture, and Treatment Protocol

1,4-Benzoquinone was obtained from Fluka AG (Buchs, Switzerland) and diethyl maleate from Sigma (St. Louis, MO). 1,4-Naphthoquinone was a generous gift from K. L. Platt (Institute of Toxicology, Mainz, FRG). The quinones were dissolved in dimethylsulfoxide immediately before use.

Chinese hamster V79 cells were maintained in Dulbecco's modified minimal essential medium supplemented with 5 or 10% fetal calf serum (5 to 10%), penicillin (100 IU/mL), and streptomycin (100 μg/mL).

Cultures of V79 cells whose medium had been changed 24 hr previously were harvested by treatment with trypsin, counted, and resuspended at a cell density of 2.5×10^7 per mL in Dulbecco's phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS), supplemented with 10 mM Hepes (pH 7.4). An aliquot (2 mL) of cell suspension was placed in a 10-mL Erlenmeyer flask. The test compound, dissolved in 10 μL dimethylsulfoxide, was added. After incubation for 1 hr at 37°C with shaking (150 rpm), the cells were washed with PBS and split. Half of the sample, in 1 mL of PBS containing 0.5 mM EDTA, was frozen and stored at −70°C for later determination of cofactor levels. The remaining cells were cultured in 150-mm dishes (seeded with 3×10^6 cells each) and 60-mm dishes (0.5 to 1×10^6 cells) for the determination of cytotoxicity and genotoxicity.

For experiments where there was previous depletion of GSH, the indicated concentrations of diethyl maleate (20 and 100 μM) were added to the culture 1 hr before harvesting. The same concentration of diethyl maleate was maintained during the exposure to 1,4-benzoquinone. Before treatment with the quinone, samples of cells were taken and frozen for the determination of the GSH level at the onset of the experiment.

Determination of the Levels of GSH, NADPH, NADH, and Their Oxidized Forms

Washed cells (2.5×10^7) were resuspended in 0.5 to 1.0 mL of sodium phosphate buffer (0.1 M, pH 7.4) and sonicated in a Branson cell disruptor B-15 at 40% pulsed duty cycles for 30 sec. The resulting cell lysate was used to determine total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) by a modification of the enzymic cycling method (*10*) as described by Hazelton and Lang (*11*). Cell homogenate was heated for 2 min in 0.1 M NaOH [for NAD(P)H] or 2 min in 0.1 M HCl [for $NAD(P)^+$] and levels of pyridine nucleotides were determined as described (*12*).

Determination of Cytotoxicity and Genotoxicity

Cytotoxicity was determined by counting the cells of two 60-mm dishes 26 hr after the exposure. For micronuclei determination, cells from one 60-mm dish were harvested 28 hr after the end of the exposure procedure. They were washed in PBS, treated for 5 min with hypotonic KCl solution (75 mM), fixed with cold methanol/acetic acid (3:1, v/v) and kept at 4°C overnight. The fixative was changed the next day. In order to achieve preparations of cells with intact cytoplasm, cell suspensions were extruded from Pasteur pipettes onto glass slides and allowed to air dry. Three days later the cells were stained in 5% Giemsa solution. Two thousand cells per data point were scored for micronuclei under light microscopy.

For determination of sister chromatid exchange, 5-bromo-2'-deoxyuridine (10 μM final concentration) was added to one 60-mm dish. Colcemid (0.2 μg/mL) was added 24 hr later. The cells were harvested after an additional 4 hr, washed in PBS, treated for 20 min with hypotonic KCl solution (75 mM), and fixed in cold methanol/acetic acid (3:1, v/v). On the following day, the fixative was changed and metaphases were prepared by dropping the cell suspension on cold, dry slides. Two days later, the slides were stained with the fluorescence dye Hoechst 33258 (25 μg/mL) for 20 min, exposed to UV light for 20 min, incubated for 2 hr in citrate buffer at 60°C, and stained with 5% Giemsa solution for 15 min. A total of 30 metaphases was scored for sister chromatid exchanges per data point.

Acquisition of 6-thioguanine resistance was used as a marker for the induction of gene mutations. For expression of the new genotype, cells in 150-mm dishes were cul-

tured for 6 days in normal medium, with one subculture in the middle of this period. After the expression time, they were replated at a density of 10^6 per 150-mm dish in medium containing 6-thioguanine (7 μg/mL) for the selection of the mutants (6 replicate plates) and at a density of 100 cells per 60-mm dish in medium without 6-thioguanine for the determination of the cloning efficiency (3 replicate plates). The plates were fixed and stained and the colonies were counted after about 7 days (cloning efficiency) or 10 days (6-thioguanine resistance).

Results

Concentration Dependence of the Effects of 1,4-Benzoquinone

1,4-Benzoquinone induced a 100-fold increase in the mutation frequency, from 4 to 415 × 10^{-6} (Table 1). This effect is similar to that observed previously (*1*); however, substantially higher concentrations were required in the present study. This may be explained by differences in the protocols related to the duration of the exposure (1 hr versus 24 hr in the previous study) and cell density (2.5 × 10^7 versus 1.5 × 10^5/mL) during the exposure. (The cell density may be important, since 1,4-benzoquinone accumulates in the cells, and since the cells may detoxify part of the test compound.) Mutagenicity was detected even at the lowest concentration (2.5 μM), but the effect per concentration unit appeared to be lower than at high exposure concentrations.

Induction of micronuclei and cytotoxicity were observed only at higher concentrations, 35 and 100 μM, respectively. Moreover, the maximum increase in the frequency of micronucleated cells above control (2.5-fold) was much weaker than the increase in the frequency of 6-thioguanine-resistant cells (100-fold). No effect on sister chromatid exchange was detected at any concentration level.

Appreciable effects on the cofactors were seen only at cytotoxic concentrations of 1,4-benzoquinone (≥ 100 μM), which were about 40 times higher than those required for detectable induction of mutations (Table 2). At these concentrations, the contents of reduced cofactors, GSH, NADPH, and NADH, were markedly decreased, and the levels of the oxidized cofactors, GSSG, $NADP^+$ and NAD^+, were increased. In the case of the pyridine cofactors, the increases in the levels of the oxidized form accounted for ≥ 50% of the missing reduced cofactors; the additional GSSG could explain only about 5% of the loss of GSH. The decrease in the GSH level cannot be attributed to unspecific leakage of the plasma membrane, since then a similar loss of NAD(H) and NADP(H) would be expected to occur. It appears more likely that GSH was conjugated with quinone.

It has to be emphasized that observable effects on the cofactors required much higher concentrations than for the induction of mutations. Even cytotoxicity did not appear to result mainly from disturbance in the cofactor levels, since their impairment at the LC_{50} (approximately 100 μM) was minimal.

Effects of 1,4-Naphthoquinone

1,4-Naphthoquinone did not induce gene mutations (Table 3). Apart from this, it was similar in its effects to 1,4-benzoquinone, even with respect to the concentrations required (Tables 3 and 4).

Cytotoxicity and Genotoxicity of 1,4-Benzoquinone in GSH-Depleted Cells

Pretreatment of V79 cells with diethyl maleate at concentrations of 20 and 100 μM led to a decrease in the GSH

Table 1. Cytotoxicity and genotoxicity of 1,4-benzoquinone in V79 cells as functions of the exposure concentration.[a]

Concentration of 1,4-benzoquinone, μM	Relative cell number, %[b]	Number of SCE per metaphase, mean ± SD[c]	Frequency of micronucleated cells, × 10^{3}[c]	Frequency of 6-thioguanine-resistant cells, × 10^{6}[d]	Mutagenicity per concentration unit[e]
0	100	7.08 ± 2.83	16	4.0	NA
2.5	116	7.15 ± 3.90	17.5	7.9	1.6
5.0	115	7.54 ± 2.47	18	15.5	2.3
10	108	7.25 ± 3.39	16	34.0	3.0
20	114	7.54 ± 2.86	19	91.3	4.4
35	107	7.37 ± 3.30	29	282.3	7.9
50	100	7.93 ± 2.70	40	248.0	4.9
100	46	7.61 ± 3.08	32	415.3	4.1
200	11	ND	ND	ND	ND
500	<2[f]	ND	ND	ND	ND

[a]Cells in suspension (5 × 10^7 cells/2 mL PBS) were exposed to the test compound for 1 hr at 37°C. Afterwards, cofactor levels (Table 2) and, after culture for appropriate expression periods, various parameters for cytotoxicity (column 2) and genotoxicity (columns 3 to 5) were determined. NA, not applicable; ND, not determined due to excessive cytotoxicity.

[b]After the exposure, 3 × 10^5 cells and 4 mL medium were put into 60-mm Petri dishes. The attached cells were harvested and counted 26 hr later. Control cultures contained 7 × 10^5 cells.

[c]Determined 28 hr after the exposure. Totals of 30 and 2000 cells per data point were scored for sister chromatid exchange and micronuclei, respectively.

[d]Determined after an expression period of 6 days.

[e]Frequency of mutants in treatment group minus frequency of mutants in control culture, × 10^6, divided by exposure concentration (in μM units).

[f]Detection limit.

Table 2. Effects of 1,4-benzoquinone on cofactor levels in V79 cells as functions of the exposure concentration.[a]

Concentration of 1,4-benzoquinone, μM	Cofactor concentrations, pmole/10^6 cells						Redox state		
	GSH	GSSG[b]	NADPH	$NADP^+$	NADH	NAD^+	GSSG[b]/GSH	$NADP^+$/NADPH	NAD^+/NADH
0	1320	49	105.6	39.6	237	82	0.037	0.375	0.346
10	1290	52	102.0	39.6	230	82	0.040	0.388	0.357
20	1300	52	104.4	42.0	232	96	0.040	0.402	0.414
50	1480	65	94.8	44.4	203	110	0.044	0.468	0.542
100	840	79	69.6	51.6	171	125	0.094	0.741	0.730
200	580	93	52.8	61.2	139	126	0.160	1.159	0.906
500	420	96	48.0	74.4	121	136	0.229	1.550	1.124

[a]Cells in suspension (5×10^7 cells/2 mL PBS) were exposed to the test compound for 1 hr at 37°C. Afterwards, cofactor levels (this table) and various parameters for cytotoxicity and genotoxicity (Table 1) were determined. However, the cofactor levels were not determined in the 2.5-, 5.0-, and 35-μM treatment groups.
[b]Expressed as molar equivalents of GSH.

Table 3. Cytotoxicity and genotoxicity of 1,4-naphthoquinone in V79 cells as functions of the exposure concentration.[a]

Concentration of 1,4-naphthoquinone, μM	Relative cell number, %[b]	Number of SCE per metaphase, mean ± SD[c]	Frequency of micronucleated cells, $\times 10^{3}$[c]	Frequency of 6-thioguanine-resistant cells, $\times 10^{6}$[d]
0	100	7.67 ± 2.88	6	12.4
2.5	111	8.63 ± 3.76	10.5	4.8
5.0	104	7.06 ± 2.86	8.5	NT
10	92	7.61 ± 3.18	11	4.4
20	51	8.46 ± 3.71	18.5	12.7
35	49	7.88 ± 2.57	9.5	9.2
50	24	8.61 ± 2.80	17.5	4.8
100	3	8.55 ± 2.91	24.5	15.2
200	<2[e]	ND	ND	ND
350	<2[e]	ND	ND	ND
500	<2[e]	ND	ND	ND

[a]Cells in suspension (5×10^7 cells/2 mL PBS) were exposed to the test compound for 1 hr at 37°C. Afterwards, cofactor levels (Table 4) and, after culture for appropriate expression periods, various parameters for cytotoxicity (column 2) and genotoxicity (column 3 to 5) were determined. NT, not tested; ND, not determined due to excessive cytotoxicity.
[b]After the exposure, 5×10^5 cells and 4 mL medium were put into 60-mm Petri dishes. The attached cells were harvested and counted 26 hr later. Control cultures contained 11×10^5 cells.
[c]Determined 28 hr after the exposure. Totals of 30 and 2000 cells per data point were scored for sister chromatid exchange and micronuclei, respectively.
[d]Determined after an expression period of 6 days.
[e]Detection limit.

Table 4. Effects of 1,4-naphthoquinone on cofactor levels in V79 cells as functions of the exposure concentration.[a]

Concentration of 1,4-naphthoquinone, μM	Cofactor concentrations, pmole/10^6 cells						Redox state		
	GSH	GSSG[b]	NADPH	$NADP^+$	NADH	NAD^+	GSSG[b]/GSH	$NADP^+$/NADPH	NAD^+/NADH
0	1060	47	90.0	36.4	163	93	0.044	0.404	0.570
5	1040	43	90.0	35.5	162	90	0.041	0.394	0.555
10	1090	46	89.5	42.7	169	87	0.042	0.477	0.514
20	1000	53	86.4	39.6	156	93	0.053	0.458	0.596
35	870	48	85.9	36.7	160	98	0.055	0.427	0.612
50	660	49	75.1	40.0	146	98	0.074	0.533	0.671
100	540	53	67.9	47.2	139	106	0.098	0.695	0.763
200	440	59	60.0	48.7	115	121	0.134	0.812	1.052
350	310	61	47.2	52.3	107	121	0.197	1.108	1.131
500	260	63	43.9	54.4	100	121	0.242	1.239	1.210

[a]Cells in suspension (5×10^7 cells/2 mL PBS) were exposed to the test compound for 1 hr at 37°C. Afterwards, cofactor levels (this table) and various parameters for cytotoxicity and genotoxicity (Table 3) were determined. However, the cofactor levels were not determined in the 2.5-μM treatment group.
[b]Expressed as molar equivalents of GSH.

level to 19 and 4% of the control value. The treatment in itself was weakly cytotoxic, but did not produce a significant induction of micronuclei or mutations. (The differences from the values of the untreated cultures were within the usual variation in controls.) 1,4-Benzoquinone showed enhanced cytotoxicity in these GSH-depleted cells (Table 5). The effect was similar at both concentration levels of diethyl maleate and equivalent to an approximately 2-fold increase in the concentration of 1,4-benzoquinone. GSH depletion had no detectable effect on the induction of micronuclei and had little effect on the induction of gene mutations by 1,4-benzoquinone. At low mutagen concentrations, the mutation frequency in the cells pretreated with diethyl maleate at the high concentration was higher than in the unpretreated cells. However, the difference may be within normal variation and, in any case, would be equivalent to a less than 2-fold increase in the 1,4-benzoquinone concentration.

Discussion

In the present study it has been shown that 1,4-benzoquinone induces gene mutations in V79 cells at normal cofactor levels. This result implies that a) the mutations are not due to disturbances in the cofactor levels. The same conclusion can be drawn from the lack of mutagenicity of 1,4-naphthoquinone and diethyl maleate, compounds which led to profound changes in cofactor levels; and b) GSH, at physiological levels, does not fully protect the cells against the mutagen. The results do not rule out partial detoxification, which may even be gathered from the hyperlinear concentration-response curve and from the loss of GSH. However, as the mutagenicity was similar in cells with very different GSH levels, nonenzymatic detoxification [being of first order for GSH (*8*)] appears to be unimportant. Enzymatic detoxification would be compatible with our results, provided the K_m for GSH was markedly below its physiological concentration.

From the diverging results with 1,4-benzoquinone and 1,4-naphthoquinone, it may be inferred that the inductions of gene mutations and micronuclei are mechanistically independent effects. However, as for the gene mutations, disturbances in cofactor levels are neither sufficient nor required for the formation of micronuclei. Moreover, over wide ranges of cellular GSH levels, similar numbers of micronuclei were induced by 1,4-benzoquinone, implying that the micronucleus-inducing species (which may be identical to or may be different from the gene mutation-inducing species) is not detoxified in a reaction of first order for GSH.

1,4-Benzoquinone and 1,4-naphthoquinone led to similar, weak increases in the GSSG level. If one accepts that this effect reflects detoxification of active oxygen, then it follows that the gene mutations, observed in 1,4-benzoquinone-treated cells, were not elicited by active oxygen. Elsewhere, 1,4-naphthoquinone should have been mutagenic as well. Active oxygen is, however, a possible candidate for the micronucleus-inducing species.

The third biological effect studied was cytotoxicity. Here again, the comparison of the active concentrations argues against the hypothesis that the toxicity results from thiol depletion and/or from pyridine cofactor oxidation. In contrast to our findings in V79 cells, cytotoxicity of menadione and 1,4-benzoquinone in isolated rat hepatocytes was observed only after the cellular defense mechanism had become exhausted and the cofactor levels were impaired (*7–9*). For examples, cytotoxicity of 1,4-benzoquinone and its methylated congeners did not occur unless the hepatocyte GSH levels were depleted by at least 90 to 95%. A possibly important difference between the cell systems is that hepatocytes are resting cells, whereas V79 cells proliferate rapidly. In addition, the measure for cytotoxicity was different. What was determined in the hepatocytes was the immediate effect, reflected in the permeability of the plasma membrane to trypan blue. In contrast, our experiments included delayed cell killing, and even inhibition of cell proliferation. While depletion of GSH was not the actual cause of

Table 5. Effects of previous GSH-depletion on cytotoxicity and genotoxicity of 1,4-benzoquinone in V79 cells.[a]

Concentration of 1,4-benzoquinone, μM	Relative cell number,[b] % B (%A)			Frequency of micronucleated cells, × 10^3[c]			Frequency of 6-thioguanine-resistant cells, × 10^6[d]		
	Control cells	20 μM DEM	100 μM DEM	Control cells	20 μM DEM	100 μM DEM	Control cells	20 μM DEM	100 μM DEM
0	100	100 (81)	100 (67)	9	9	13	1	7	4
10	100	87 (70)	99 (66)	18	19	23	27	29	50
35	95	58 (47)	62 (42)	18	34	28	196	129	249
50	53	39 (31)	40 (27)	40	34	28	392	426	383
100	47	20 (16)	20 (13)	46	47	43	404	624	517
200	18	6 (5)	2 (2)	40	ND	ND	545	ND	ND

[a]Cells were treated for 1 hr with diethyl maleate (DEM) at concentrations of 0, 20, and 100 μM. At this time point, they contained 1.06, 0.20, and 0.04 nmole GSH/10^6 cells, respectively. Cells in suspension (5×10^7 cells/2 mL PBS) were then exposed to 1,4-benzoquinone. After culture for appropriate expression periods, cytotoxic and genotoxic effects were determined. ND, not determined due to excessive cytotoxicity.

[b]After the exposure, 10^6 cells and 4 mL medium were put into 60-mm Petri dishes. The attached cells were harvested and counted 26 hr later. Untreated cultures (control A) contained 2.4×10^6 cells. The controls B were not exposed to 1,4-benzoquinone, but were exposed to diethyl maleate at the corresponding concentration.

[c]Determined 28 hr after the exposure. A total of 2000 cells was scored for micronuclei per data point.

[d]Determined after an expression period of 6 days.

1,4-benzoquinone cytotoxicity, previous depletion of GSH with diethyl maleate enhanced this effect. Inhibition of quinone detoxification may be involved in this synergism, but additional mechanisms cannot be ruled out.

In summary, 1,4-benzoquinone is a potent mutagen in mammalian cells. Mutagenic effects occur at low, virtually noncytotoxic concentrations. The mutagenicity does not result from disturbance of cofactor levels, nor do reactive forms of oxygen appear important. Exhaustion of a detoxification system, which would imply a threshold concentration, is not required. These findings support the hypothesis that 1,4-benzoquinone and 1,4-benzohydroquinone, which shows similar mutagenic effects in V79 cells (*1*), are toxicologically important metabolites of benzene.

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