Free Rad. Res. Comms., Vol. 8, No. 4-6, pp. 355-363
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PYRIDINE NUCLEOTIDE CHANGES IN HEPATOCYTES EXPOSED TO QUINONES

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(Received June 20, 1989; accepted July 11, 1989)

Quinones may be toxic by a number of mechanisms, including arylation and oxidative stress caused by redox cycling. Using isolated hepatocytes, we have studied the cytotoxicity of four quinones, with differing abilities to arylate cellular nucleophiles and redox cycle, in relation to their effects on cellular pyridine nucleotides. High concentrations of menadione (redox cycles and arylates), 2-hydroxy-1,4-naphthoquinone (neither arylates nor redox cycles via a one electron reduction) 2,3-dimethoxy-1,4-naphthoquinone (a pure redox cycler) and p-benzoquinone (a pure arylator) caused an initial decrease in NAD^+ and loss of viability, which was not prevented by 3-aminobenzamide, an inhibitor of poly(ADP-ribose)polymerase. In contrast, 3-aminobenzamide inhibited the loss of NAD^+ and viability caused by dimethyl sulphate so implicating poly(ADP-ribose)polymerase in its toxicity but not that of the quinones. Non-toxic concentrations of menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone all caused markedly similar changes in cellular pyridine nucleotides. An initial decrease in NAD^+ was accompanied by a small, transient increase in $NADP^+$ and followed by a larger, prolonged increase in NADPH and total $NADP^+$ + NADPH. Nucleotide changes were not observed with non-toxic concentrations of p-benzoquinone. Our findings suggest that a primary event in the response of the cell to redox cycling quinones is to bring about an interconversion of pyridine nucleotides, in an attempt to combat the effects of oxidative stress.

KEY WORDS: Quinones, Oxidative Stress, Poly(ADP-ribosyl)polymerase, Nucleotide Interconversion.

INTRODUCTION

There is much interest in understanding the mechanism of toxicity of quinones due to their widespread occurrence in the environment and the use of certain quinones as therapeutic agents such as the anticancer agent, adriamycin. Whilst most current data suggest that quinones may exert their toxicity by their ability to cause an oxidative stress by redox cycling,[1,2] they may also exert their toxicity by other mechanisms such as direct interaction with cellular macromolecules[3,4] or by inhibition of mitochondrial electron transport.[5]

Cellular reductases may reduce quinones either by one or two electron reduction to the corresponding semiquinones or hydroquinones respectively.[1,6] The semiquinones may be toxic per se or they may react with molecular oxygen to form superoxide anion radical and regenerate the parent quinone, which is then available to be rereduced and undergo a futile redox cycle.[1,2,6] The net result of this redox cycling is an oxidative

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stress resulting from a disproportionate consumption of cellular reducing equivalents and the generation of active oxygen species $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$). One electron reduction of quinones to semiquinones is therefore believed to result in the generation of toxic metabolites and may be carried out by a number of cellular reductases including NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH-ubiquinone oxidoreductase.[1,6,7] In contrast, the two electron reduction of the quinones to hydroquinones, catalysed by DT-diaphorase[8] is widely believed to be a detoxication reaction because hydroquinones are both generally less reactive and may also be readily conjugated and excreted.

In addition to the loss of pyridine nucleotides, catalysed by cellular reductases through one and two electron reduction, further loss of NADPH is caused by removal of H_2O_2 through the GSH peroxidase/GSSG reductase cycle.[1] Some of the NADPH is restored by activation of the hexose monophosphate shunt.

A number of studies have investigated the mechanism of cytotoxicity to isolated hepatocytes of naphthoquinones, the most extensively studied being menadione (2-methyl-1,4-naphthoquinone).[9-15] Exposure of hepatocytes to menadione results in a rapid oxidation of intracellular glutathione (GSH), with the appearance of increased levels of GSSG, oxidised protein sulphydryls and protein mixed disulphides.[9-12] This is accompanied by plasma membrane blebbing, a sustained rise in cytosolic free Ca^{2+} levels, and marked oxidation of NADPH. The elevation of cytosolic free Ca^{2+} may then cause toxicity by a number of mechanisms including activation of proteases, phospholipases or endonucleases.[13]

A more complete understanding of the mechanism of toxicity of menadione is complicated because it may cause both oxidative stress via redox cycling and/or arylation of protein thiols.[14,15] In an attempt to identify those properties of menadione responsible for its observed actions, we have compared its toxicity and its effects on nucleotide levels with those of three other quinones i.e. p-benzoquinone (a pure arylator),[16] 2,3-dimethoxy-1,4-naphthoquinone, (a pure redox cycler)[17] and 2-hydroxy-1,4-naphthoquinone, which neither arylates nor redox cycles following a one electron reduction.[18]

MATERIALS AND METHODS

2-Methyl-1,4-naphthoquinone, p-benzoquinone, 2-hydroxy-1,4-naphthoquinone and tributylamine were from Aldrich Chemical Co. Ltd (Gillingham, Dorset, UK). 2,3-Dimethoxy-1,4-naphthoquinone was synthesised as previously described.[17] Collagenase, HEPES, firefly lantern extract, thiazolyl blue, phenazine ethosulphate, alcohol dehydrogenase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase and all nucleotides were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK).

Cell isolation and incubation

Hepatocytes were prepared from male ICI Alderley Park strain Wistar rats (ICI plc, Alderley Park, Cheshire, UK) using Sigma type IV collagenase and incubated in rotating round bottomed flasks in a Krebs-Henseleit salt solution containing 12.5 mM HEPES buffer.[17] Cell viability was determined by trypan blue exclusion in a 0.2% solution and samples were prepared and stored as previously described.[19] Protein was

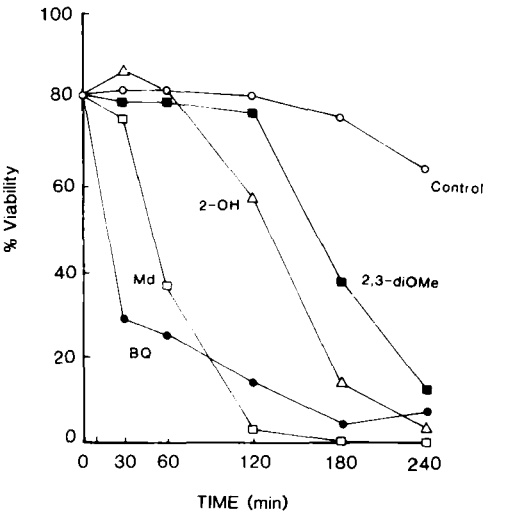

FIGURE 1 Effects of quinones on cell viability. Hepatocytes were incubated either alone (O—O) or with menadione (Md, 200 μM), p-benzoquinone (BQ, 100 μM), 2,3-dimethoxy-1,4-naphthoquinone (2,3-DiOMe, 200 μM) or 2-hydroxy-1,4-naphthoquinone (2-OH-1,4-NQ, 200 μM). Cell viability was assessed by trypan blue exclusion and the results represent the mean of at least three separate preparations.

measured by the method of Lowry *et al.*[20] Oxidized nucleotides were determined using the spectrophotometric recycling assay of Bernofsky and Swan[21] as previously described.[22] The reduced pyridine nucleotides were separated using high performance liquid chromatography on a C18 reverse-phase column using isocratic elution with 0.2 M ammonium phosphate (pH 6.0)/17.87% methanol/0.13% tributylamine.[22]

RESULTS

Effects of quinones on toxicity

The four quinones caused a concentration- and time-dependent cytotoxicity to freshly isolated hepatocytes (Figure 1). The order of toxicity was benzoquinone > menadione > 2-hydroxy-1,4-naphthoquinone > 2,3-dimethoxy-1,4-naphthoquinone.

Effects of 3-aminobenzamide on toxicity and NAD$^+$ depletion produced by the quinones and dimethyl sulphate

Pretreatment of hepatocytes with 3-aminobenzamide (20 mM), an inhibitor of poly(ADP-ribose) polymerase, delayed the toxicity associated with dimethyl sulphate and the associated decrease in NAD$^+$ (Table 1). 3-Aminobenzamide (20 mM) neither affected the loss of viability nor the decrease in cellular NAD$^+$ caused by the four quinones (Table 1).

Effects of quinones on pyridine nucleotides

Nucleotide levels were analysed following exposure to non-toxic quinone concentra-

TABLE I

Effects of 3-Aminobenzamide on the Toxicity and NAD^+-depletion caused by Dimethyl Sulphate and the Quinones

Incubation conditions	% viability		nmoles NAD^+/mg protein	
	−3AB	+3AB	−3AB	+3AB
Dimethyl Sulphate (500 μM)	17 ± 7	79 ± 8	0.15 ± 0.004	2.78 ± 0.23
Menadione (200 μM)	28 ± 5	23 ± 13	0.48 ± 0.12	0.49 ± 0.19
2,3-Dimethoxy-1,4-NQ (200 μM)	37 ± 14	34	0.33 ± 0.12	0.29
2-Hydroxy-1,4-NQ (200 μM)	14 ± 3	17	0.24 ± 0.10	0.19
P-Benzoquinone (100 μM)	4 ± 4	6	0.02 ± 0.002	0.07

Hepatocytes were incubated in the presence or absence of 3-aminobenzamide (20 mM) (3AB) for 180 mins. NAD^+ content and cell viability were determined as described in Materials and Methods. Control viability was 77 ± 3%. Control NAD^+ content was 2.90 ± 0.42 nmoles/mg protein and average protein content was 2.07 mg/10^6 cells. Values are the mean of 2 or more separate preparations.

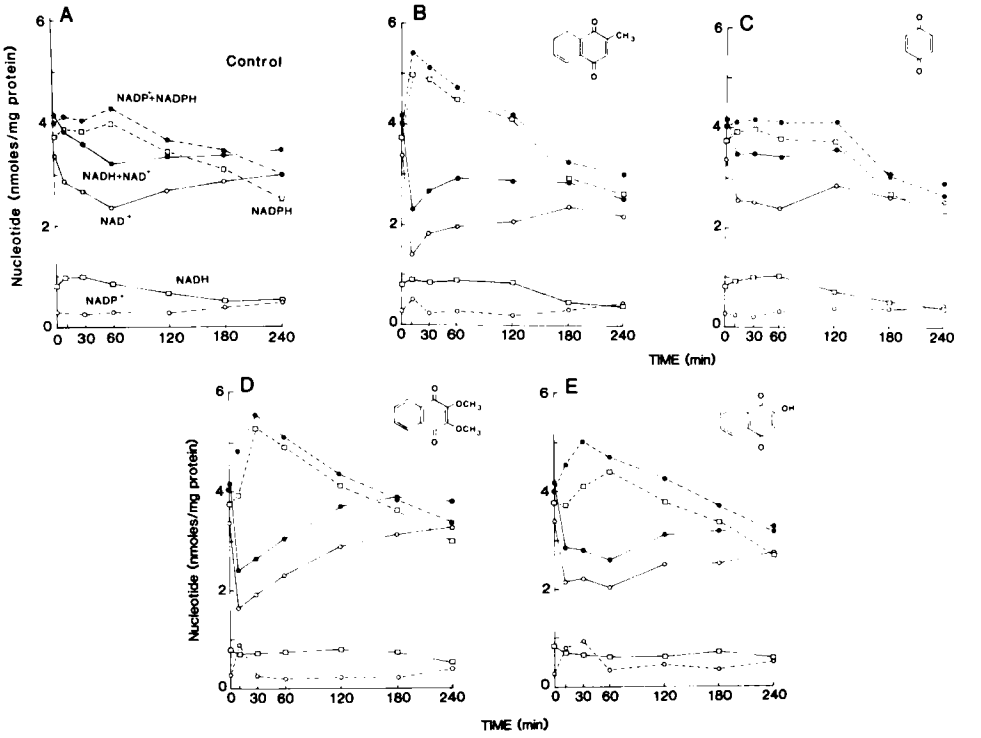

FIGURE 2 Effects of non-toxic concentrations of quinones on cellular pyridine nucleotides. Hepatocytes were incubated either A) alone or with B) menadione (50 μM), C) p-benzoquinone (20 μM), D) 2,3-dimethoxy-1,4-naphthoquinone (50 μM) or E) 2-hydroxy-1,4-naphthoquinone (50 μM). At the indicated times, intracellular NAD^+ (○—○), NADH (□—□), total NAD^+ + $NADH^+$ (●—●), $NADP^+$ (○---○), NADPH (□---□) and total $NADP^+$ + NADPH (●---●) were measured as described in Materials and Methods. The values represent the means from three separate hepatocyte preparations (average protein content = 2.03 mg/10^6 cells).

TABLE 2
Effects of non-toxic concentrations of quinones on total pyridine nucleotide levels.

	$NAD^+ + NADH$	$NADP^+ + NADPH$	Total pyridine nucleotides
Control	3.53 ± 0.23	4.05 ± 0.14	7.58 ± 0.36
Menadione (50 μM)	2.73 ± 0.41	5.13 ± 0.36	7.87 ± 0.77
2,3-dimethoxy-1,4-NQ (50 μM)	2.64 ± 0.17	5.55 ± 0.19	8.19 ± 0.06
2-Hydroxy-1,4-NQ (50 μM)	2.79 ± 0.32	4.71 ± 0.35	7.50 ± 0.41
p-Benzoquinone (20 μM)	3.50 ± 0.36	4.16 ± 0.24	7.66 ± 0.59

Nucleotide measurements (nmoles/mg protein) were made after 30 minutes incubation in the presence of the quinones as described in Materials and Methods. The values represent Mean ± S.E. from three separate hepatocyte preparations (average protein content = 2.03 mg/10^{-6} cells).

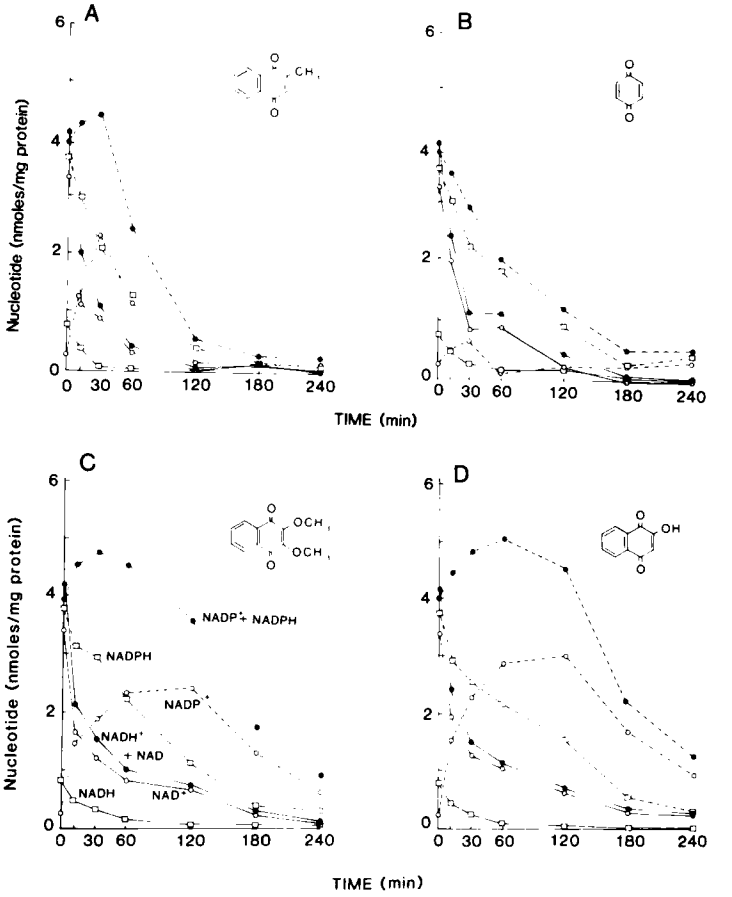

FIGURE 3 Effects of toxic concentrations of quinones on oxidized and reduced cellular pyridine nucleotides. Hepatocytes were incubated either with A) menadione (200 μM), B) p-benzoquinone (100 μM), C) 2,3-dimethoxy-1,4-naphthoquinone (200 μM) or D) 2-hydroxy-1,4-naphthoquinone (200 μM). Cellular nucleotides were measured as described in Materials and Methods. The symbols used are the same as those described in the legend to Fig. 2. The values represent the means of three separate hepatocyte preparations (average protein content = 2.03 mg/10^6 cells).

tions (Figure 2 and Table 2). A small decrease in the level of $NADP^+$ + NADPH was observed in control hepatocytes during the 4 hr incubation (Figure 2A). At a non-toxic concentration of benzoquinone (20 μM) no effect on NAD(H) or NADP(H) was observed (Figure 2C). However, non-toxic concentrations (50 μM) of menadione, 2-hydroxy- and 2,3-dimethoxy-1,4-naphthoquinone caused a prolonged increase in the levels of NADPH and total $NADP^+$ + NADPH accompanied by a small transient increase in $NADP^+$ over the first 10–30 mins of incubation (Figure 2B, D and E). The non-toxic concentrations had little or no effect on the levels of NADH but caused an initial rapid decline in the levels of NAD^+ which was slowly reversed during the course of the incubation (Figure 2). This was seen most clearly with the cells exposed to 2,3-dimethoxy-1,4-naphthoquinone (Figure 2D).

Higher toxic concentrations of menadione (200 μM), 2,3-dimethoxy-1,4-naphthoquinone (200 μM) and 2-hydroxy-1,4-naphthoquinone (200 μM), caused a rapid depletion of NAD^+ and NADH (Figure 3) which preceded cell death but appeared to accompany the loss of viability with p-benzoquinone (100 μM). However, with p-benzoquinone a very marked fall in viability was observed with only a small increase in concentration which complicated the identification of changes prior to cell death. Prior to cell death, cytotoxic concentrations (200 μM) of menadione, 2-hydroxy-1,4-naphthoquinone and 2,3-dimethoxy-1,4-naphthoquinone caused extensive oxidation of NADPH, as seen by a fall in NADPH accompanied by an increase in $NADP^+$ which was followed by a decrease in $NADP^+$ associated with cell death (Figure 3A, B, C, and D). This was most evident with the less toxic quinones i.e. 2,3-dimethoxy- and 2-hydroxy-1,4-naphthoquinone (Figure 3C and D) where the time of onset of cell death occurred later in the incubation (Figure 1). In contrast to the results with the other quinones, benzoquinone caused only a small increase in $NADP^+$ but a large decrease in NADPH (Figure 3B).

DISCUSSION

Recently Schrauffstätter *et al.*[23] have studied the cytotoxicity of H_2O_2 to a murine macrophage-like cell line. Low, non-toxic concentrations of H_2O_2 caused an early and reversible decrease in NAD^+ and ATP whereas toxic concentrations caused an irreversible depletion. The depletion of NAD^+ was apparently due to H_2O_2-induced DNA strand breaks causing an activation of poly(ADP-ribose)polymerase (EC 2.4.2.30).[24,25] Polymerase activation and NAD^+ loss are observed following exposure to a number of DNA-damaging agents, including alkylating agents and ionizing radiation.[26] Further evidence for the involvement of poly(ADP-ribose)polymerase in H_2O_2-induced toxicity was provided by the use of inhibitors of the enzyme (i.e. 3-aminobenzamide, nicotinamide or theophylline) which prevented the loss of both NAD^+ and ATP, as well as the accompanying cell death.[25]

Low concentrations of menadione (25 μM) also cause DNA strand breaks in hepatocytes.[27] We have therefore investigated a possible role for poly(ADP-ribose)polymerase in the toxicity of the quinones in hepatocytes and compared their effects to those of dimethyl sulphate, a DNA alkylating agent, which induces single strand breaks and activates poly(ADP-ribose)polymerase in a wide range of cell types.[26] The effects of this activation, i.e. DNA ligation and NAD^+ depletion, is prevented by inhibitors of poly(ADP-ribose)polymerase.

All four quinones and dimethyl sulphate produced a rapid, depletion of NAD^+,

which preceded the loss of ATP and cell viability. Inhibitors of poly(ADP-ribose)polymerase protected against the NAD^+ depletion and cytotoxicity in the cases of dimethyl sulphate (Table 1) and H_2O_2[19] but not with the quinones. The depletion of NAD^+ caused by the quinones is therefore due to a mechanism other than one involving an activation of poly(ADP-ribose)polymerase. The protection afforded by 3-aminobenzamide against both the cytotoxicity and NAD^+ depletion induced by dimethyl sulphate (Table 1) supported the involvement of the polymerase in its cytotoxicity.

Previous workers have shown that low concentrations of menadione (10–50 μM) have no effect on the NADPH/$NADP^+$ + NADPH ratio[9] and that higher, but still non-toxic concentrations (100 μM), cause an initial depletion of NADPH followed by an increase above controls.[28] The effects of the quinones on cellular pyridine nucleotides were most interesting. With both non-toxic and toxic concentrations of menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, a striking coincidence in the fall of NAD^+ with a rise in total NADP(H) was observed such that total pyridine nucleotide levels remained constant (Table 2). These results support the hypothesis that the initial NAD^+ depletion is due to an interconversion of pyridine nucleotides, with a synthesis of NADPH at the expense of NAD^+. The sequence of events may be envisaged as an initial conversion of NAD^+ to $NADP^+$ followed by a reduction of $NADP^+$ to NADPH. Synthesis of $NADP^+$ from NAD^+ could occur by a number of indirect routes, but the simplest mechanism would be mediated by NAD^+ kinase (for a fuller discussion see reference 22). The mechanism by which the quinones may activate NAD^+ kinase is unclear but may involve calmodulin.

The characteristic nucleotide changes, i.e. initial decrease in NAD^+ and a small transient increase in $NADP^+$ followed by a more prolonged and extensive increase in NADPH, induced by non-toxic concentrations of 2,3-dimethoxy-1,4-naphthoquinone, menadione and 2-hydroxy-1,4-naphthoquinone (Figures 2B, D and E) appear to be changes indicative of oxidative stress. These results would be expected from 2,3-dimethoxy-1,4-naphthoquinone and menadione both of which are good redox cyclers,[9–13,15,17] although the toxicity of menadione may also be due at least in part to arylation reactions.[14,15,17] However most surprising were the results with 2-hydroxy-1,4-naphthoquinone which is a very poor substrate for cellular one electron reductases such as NADPH-cytochrome P450 reductase, in part because of its very low one electron reduction potential (E_7^1-415 mV).[18] Thus 2-hydroxy-1,4-naphthoquinone induces an oxidative stress in hepatocytes similar to that observed by other quinones, while its mechanism of activation appears to be different from many other quinones. Preliminary results suggest that it may be activated by a two electron reduction catalysed by DT-diaphorase to its hydroquinone, which then rapidly autooxidizes producing hydrogen peroxide and inducing an oxidative stress (Gant, Stubberfield and Cohen — unpublished results).

In summary, menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone cause characteristic changes in cellular pyridine nucleotides in hepatocytes indicative of oxidative stress and were not observed with p-benzoquinone, which acts by arylation. These changes, including an interconversion of pyridine nucleotides, followed by a more prolonged elevation in NADPH, increase the ability of the cell to cope with an oxidative stress. This may be a general mechanism by which cells cope with an oxidative stress.

Acknowledgements

This work was supported in part by a grant from the Wellcome Trust. CRS was in receipt of a M.R.C. Studentship. We thank Mrs M. Fagg for the typing of the manuscript and Mr D. King for preparation of the figures.

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Accepted by Prof. H. Sies/Prof. E. Cadenas