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PYRIDINE NUCLEOTIDE HYDROLYSIS AND INTERCONVERSION IN RAT HEPATOCYTES DURING OXIDATIVE STRESS

WINSTON A. MORGAN*

Interdisciplinary Centre for Cell Modulation Studies, University of East London, Romford Road, London E15 4LZ, U.K.

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Abstract—A characteristic feature of many types of chemically induced oxidative stress is a depletion of the pyridine nucleotide NAD^+. This has been attributed to either its hydrolysis to nicotinamide and ADP-ribose or to its phosphorylation (interconversion) to $NADP^+$. In this study the exposure of rat hepatocytes to either *tert*-butyl hydroperoxide (250–750 μM) or 2,3-dimethoxy-1,4-naphthoquinone (50 μM) resulted in a rapid depletion of NAD^+ with no change in the level of NAD. The depletion of NAD^+ was accompanied by an increase in nicotinamide. The rate of NAD^+ deletion induced by *tert*-butyl hydroperoxide (500 μM) and 2,3-dimethoxy-1,4-naphthoquinone (50 μM) was reduced by preincubating the hepatocytes for 1 hr with either 3-aminobenzamide (20 mM), nicotinamide (10 mM) or theophylline (7.5 mM), potent inhibitors of poly(ADP-ribose)polymerase. In cells exposed to 2,3-dimethoxy-1,4-naphthoquinone (50 μM) extensive oxidation of NADPH to $NADP^+$ was observed; this was followed by an increase in the level of $NADP^+$ + NADPH (NADP(H)). However, no change in the total pyridine nucleotide (NAD(H) + NADP(H)) pool was detected. Exposure to *tert*-butyl hydroperoxide resulted in the oxidation of NADPH to $NADP^+$ and a decrease in total pyridine nucleotide pool. These results suggest that during oxidative stress, NAD^+ is hydrolysed to nicotinamide, possibly by the activation of poly(ADP-ribose)polymerase and that the depletion of NAD^+ is independent of the increase in $NADP^+$. Furthermore, no evidence of an interconversion of NAD^+ to $NADP^+$ was found.

Key words: pyridine nucleotide; poly(ADP-ribose) polymerase; interconversion redox cycling; oxidative stress; inhibitors

In rat hepatocytes, pyridine nucleotides exist in two main forms: NADPH, which is located primarily in the mitochondria; and NAD^+ which is found predominantly in the extramitochondrial compartments [1]. Alterations in the level and redox status of cellular pyridine nucleotides have been reported in hepatocytes exposed to redox cycling quinones and organic hydroperoxides and have been implicated in oxidant-induced cell death [2, 3]. The enzymic reduction of quinones either by a one or two electron reduction may alter the redox status of NADP(H) ($NADP^+$ + NADPH) in favour of $NADP^+$. A similar alteration in the redox status of pyridine nucleotides will result from the metabolism of tBH† following the activation of the GSH peroxidase/GSSG reductase system [4]. Changes in the level and redox status of NAD(H) (NAD^+ + NADH) are also observed during oxidative stress [5]. The depletion of NAD^+ is often associated with DNA damage and the activation of poly(ADP-ribose)polymerase, which hydrolyses NAD^+ to nicotinamide and ADP-ribose [6].

In a number of recent studies on rat hepatocytes, an interconversion of NAD(H) to NADP(H) was proposed to explain the depletion of NAD^+ observed during oxidative stress induced several redox cycling quinones [2, 7]. Furthermore, in rat hepatocytes exposed to tBH, Yamamoto *et al.* proposed an interconversion of NAD^+, NADH and NADPH to $NADP^+$ [8]. In both studies, the depletion of NAD^+ was not prevented by 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose)polymerase and the depletion of NAD(H) was accompanied by an increase in NADP(H) [2, 7, 8]. In contrast, in P388D_1 cells, the depletion of NAD^+ induced by hydrogen peroxide was prevented by various inhibitors of poly(ADP-ribose)polymerase [9]. Similarly, with 1C1C7 hepatoma cells exposed to the redox cycling quinone 2,3-diOMe-1,4-NQ a rapid depletion of NAD^+ was observed, which was prevented by 3-aminobenzamide [10].

In order to resolve these differences, pyridine nucleotide changes and the formation of nicotinamide was determined in rat hepatocytes exposed to menadione, 2,3-diOMe-1,4-NQ and tBH in the absence and presence of inhibitors of poly(ADP-ribose)polymerase. Nicotinamide formation was accompanied by a rapid depletion of NAD^+ and reduced by preincubating the cells with inhibitors of poly(ADP-ribose)polymerase. These results suggested that the changes in pyridine nucleotides observed during oxidative stress were mainly due to alterations in the redox status of NADP(H) and the hydrolysis of NAD^+ to nicotinamide and ADP-ribose rather than an interconversion of NAD(H) to NADP(H).

* Correspondence. Tel. 0181-590 7722.

†Abbreviations: tBH, *tert*-butylhydroperoxide; GSH, glutathione; GSSG, glutathione disulphide; 2,3-diOMe-1,4-NQ, 2,3-dimethoxy-1,4-naphthoquinone.

MATERIALS AND METHODS

Materials. Collagenase type 1, HEPES, thiazolyl blue, phenazine ethosulphate, alcohol dehydrogenase, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-aminobenzamide, nicotinamide, theophylline and all nucleotides were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). Tributylamine was purchased from Aldrich Chemical Co. (Gillingham, U.K.). 2,3-diOMe-1,4-NQ was synthesized as previously described [2]. All other chemicals were purchased from BDH Ltd (Poole, Dorset, U.K.).

Isolation and incubation of rat hepatocytes. Hepatocytes were prepared as previously described [11] by collagenase perfusion of the livers of adult male Wistar rats (220–260 g). The isolated cells (10^6 cell/mL) were suspended in Krebs Henseleit buffer containing 12.5 mM HEPES under an atmosphere of 95% O_2/5% CO_2 (pH 7.4) and incubated in continuously rotating round-bottomed flasks at 37° for 30 min. Cell viability was determined by trypan blue exclusion in a 0.4% (w/v) solution and samples were prepared as previously described [2]. Protein was measured by the method of Lowry [12].

Determination of pyridine nucleotides. Oxidized pyridine nucleotides (NAD^+, $NADP^+$) were determined using the spectrophotometric recycling assay of Bernofsky and Swan [13] as previously described [2]. Reduced pyridine nucleotides were first separated by HPLC on a C18 reverse-phase Bondapak column (Millipore Ltd, Hertfordshire, U.K.) using an isocratic gradient with 82% 0.2 mM ammonium phosphate (pH 6.0)/17.85% methanol/0.15% tributylamine as described previously [2, 14]. The eluent was monitored at 340 nm and concentration determined by peak height.

Inhibition of poly(ADP-ribose)polymerase. Freshly isolated hepatocytes were incubated for one hour with either 3-aminobenzamide (20 mM), nicotinamide (10 mM) or theophylline (7.5 mM) to increase the intracellular concentration of the inhibitors. The cells were then exposed to either tBH (500 μM) or 2,3-diOMe-1,4-NQ (50 μM) and aliquots of cell suspension were removed for analysis of NAD^+.

Determination of NAD^+ hydrolysis. NAD^+ hydrolysis was determined by measuring the simultaneous depletion of NAD^+ and the increase in nicotinamide. Intracellular NAD^+ was radioactively labelled in the nicotinamide moiety following intraperitoneal injection of male Wistar rat (200–230 g) with 12.5 μCi (0.23 μmol) [carbonyl-^{14}C]-nicotinamide (Amersham Ltd, Buckinghamshire, U.K.) as described previously [15]. After 4–5 hr hepatocytes were isolated as described above. Following a 30 min preincubation, the cells were exposed to either tBH (500 μM), 2,3-diOMe-1,4-NQ (50 μM), menadione (50 μM) or dimethyl sulphate (800 μM) and at various times aliquots were removed for analysis of NAD^+ and nicotinamide.

NAD^+ and nicotinamide were extracted by resuspension of 10^6 cells in 1.0 mL 0.5 M perchloric acid as described previously [16]. Prior to analysis the acid extracts were partially neutralized by the addition of 50 μL KOH (3 M) and 10 μL KH_2PO_4 (0.1 M) to 200 μL of the perchloric acid extract. NAD^+ and nicotinamide were first separated by ion-exchange HPLC on a partisil SAX ion-exchanger (Millipore) attached to the System Gold HPLC system (Beckman Ltd, Buckinghamshire, U.K.) and a Waters 484 UV detector (Waters, Millipore, Hertfordshire, U.K.) set at 254 nm. Optimum separation was achieved using an isocratic gradient with 0.01 M ammonium phosphate (pH 4.9) pumped at 1.0 mL/min for 10 min, followed by 0.2 M ammonium phosphate (pH 4.8) at 1.0 mL/min for 10 min, finally 0.01 M ammonium phosphate (pH 4.9) at 1.0 mL/min for 25 min. Peaks containing NAD^+ and nicotinamide were identified by injecting unlabelled NAD^+ and nicotinamide and comparing retention times. Radioactivity was determined by liquid scintillation counting using aquasol scintillation fluid (Dupont Ltd, Hertfordshire, U.K.).

Statistical analysis. The results were expressed as mean ± SEM. Where appropriate statistical significance was tested by Student's *t*-test with the accepted significance level of $P < 0.05$.

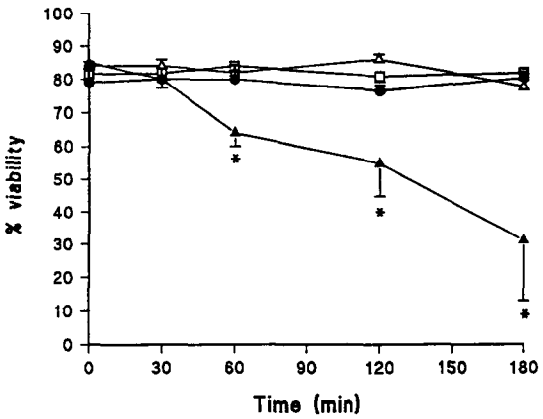


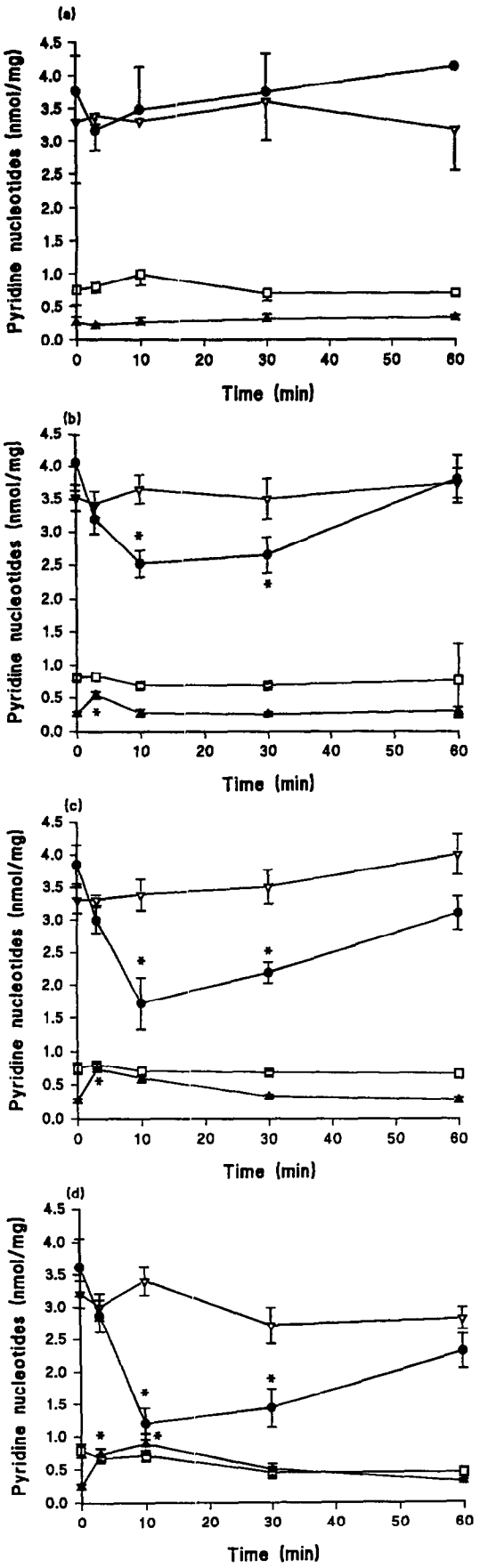
Fig. 1. The effects of tBH on the viability of rat hepatocytes. Cells were incubated either (△) alone or with tBH: (□) 250 μM, (●) 500 μM, or (▲) 750 μM. Cell viability was determined by trypan blue exclusion as described in the Materials and Methods. Values represent mean ± SEM of three separate incubations. *Significantly different ($P < 0.05$) from the control.

RESULTS

Effect of tert-*butyl hydroperoxide on cellular viability and the pyridine nucleotides*

No loss in cell viability was observed in hepatocytes exposed to low concentrations of tBH (250–500 μM) (Fig. 1). Higher concentrations (750 μM) caused a loss of membrane integrity, which was detected within 60 min (Fig. 1).

Similar changes in intracellular pyridine nucleotides were observed in hepatocytes exposed to toxic and non-toxic concentrations of tBH (Fig. 2). Within the first 30 min of exposure to tBH, a concentration dependent increase in the level of $NADP^+$ was detected; however no change in the level of NADPH was observed (Fig. 2(b)–(d)). Whereas tBH induced no change in the level of NADH, a rapid



concentration dependent depletion of NAD^+ was observed within 10 min of exposure to the hydroperoxide (Fig. 2(b)–(d)). The depletion of NAD^+ was not accounted for by an increase in the level of NADP(H). Furthermore the depletion of NAD^+ was accompanied by a decrease in total pyridine nucleotides (Table 1).

The effect of 2,3-diOMe-1,4-NQ on the pyridine nucleotides

As previously reported [2], 2,3-diOMe-1,4-NQ (50 μM) caused an initial decrease in NAD^+ and a transient increase in $NADP^+$, followed by a prolonged increase in NADP(H) (Fig. 3). No change in NADH was detected (Fig. 3). By measuring the nucleotides after 3 min exposure, an initial decrease in NADPH was observed. This was in agreement with the previously reported early decrease in NADPH in hepatocytes exposed to menadione [17] and may account for the early increase in $NADP^+$. In agreement with our previous observations [2], the decrease in NAD(H) observed at 30 min was approximately equal to the increase in NADP(H) and no significant change in the total pyridine nucleotide pool was observed (Table 1).

Inhibition of poly(ADP-ribose)polymerase

The evidence for nucleotide interconversion was partially based on the depletion of NAD^+ in the presence of the poly(ADP-ribose)polymerase inhibitor, 3-aminobenzamide [8, 16]. In an attempt to ascertain further the role of poly(ADP-ribose)polymerase in the depletion of NAD^+, hepatocytes were preincubated for 1 hr with either 3-aminobenzamide (20 mM), nicotinamide (10 mM) or theophylline (7.5 mM). In the absence of the polymerase inhibitors, intracellular NAD^+ was 3.9 ± 0.2 nmol/mg protein; in the presence of nicotinamide, 3-aminobenzamide and theophylline intracellular NAD^+ was 5.0 ± 0.2, 4.4 ± 0.3 and 3.7 ± 0.2 nmol/mg protein, respectively.

In the presence of the polymerase inhibitors, the rate of NAD^+ depletion induced by 2,3-diOMe-1,4-NQ and tBH fell significantly (Table 2). As preincubation with the polymerase inhibitor alters the level of the cellular NAD^+, the percentage of total cellular NAD^+ depleted by 2,3-diOMe-1,4-NQ and tBH in the absence and presence of the inhibitor was investigated. In cells exposed to 2,3-diOMe-1,4-NQ (50 μM) as well as to tBH (500 μM), a large percentage of total cellular NAD^+ was depleted (Table 3). In the presence of the polymerase inhibitors, significantly less of the total cellular NAD^+ was depleted by either 2,3-diOMe-1,4-NQ (Table 3) or by tBH (Table 3). In preliminary studies where the preincubation period was less than 60 min, no inhibition of the depletion of NAD^+ was observed.

Fig. 2. The effects of tBH on cellular pyridine nucleotides. Hepatocytes were incubated either (a) alone, or with tBH: (b) 250 μM, (c) 500 μM, or (d) 750 μM. At the times indicated intracellular (●) NAD^+, (□) NADH, (▲) $NADP^+$, and (▽) NADPH were determined as in Materials and Methods. Values represent mean ± SEM of three separate incubations. *Significantly different ($P < 0.05$) from the control.

Table 1. Effect of 2,3-diOMe-1,4-NQ and tBH on total pyridine nucleotide levels

	$NAD^+ + NADH$ {NAD(H)}	$NADP^+ + NADPH$ {NADP(H)}	Total pyridine nucleotides
Control	4.45 ± 0.57	3.95 ± 0.59	8.60 ± 0.80
2,3-diOMe-1,4NQ (50 μM)	3.30 ± 0.16	5.32 ± 0.66	8.63 ± 0.78
tBH (250 μM)	3.34 ± 0.26	3.76 ± 0.30	7.10 ± 0.53
tBH (500 μM)	2.77 ± 0.16*	3.80 ± 0.26	6.59 ± 0.41
tBH (750 μM)	1.92 ± 0.28†	3.27 ± 0.23	5.19 ± 0.38†

Nucleotides measurements (nmol/mg protein) were made after 30 min incubation in the presence of either 2,3-diOMe-1,4-NQ or tBH as described in Material and Methods. The values represent mean ± SEM from three separate hepatocyte preparations.

* P (0.05) significantly less than control.

† P (0.02) significantly less than control.

The hydrolysis of NAD^+

Poly(ADP-ribose)polymerase is well known to hydrolyse NAD^+ to nicotinamide and ADP-ribose [6]. A depletion of NAD^+ accompanied by increased nicotinamide formation would strongly suggest the enzymic hydrolysis of NAD^+ by the polymerase. After 15 and 30 min, a simultaneous depletion of NAD^+ and increase in nicotinamide was observed (Fig. 4). The increase in nicotinamide was approximately equal to the NAD^+ loss. The alkylating agent, dimethyl sulphate, which is known to activate poly(ADP-ribose)polymerase [18], also caused a simultaneous depletion of NAD^+ and increased nicotinamide formation in rat hepatocytes (Fig. 4(b)).

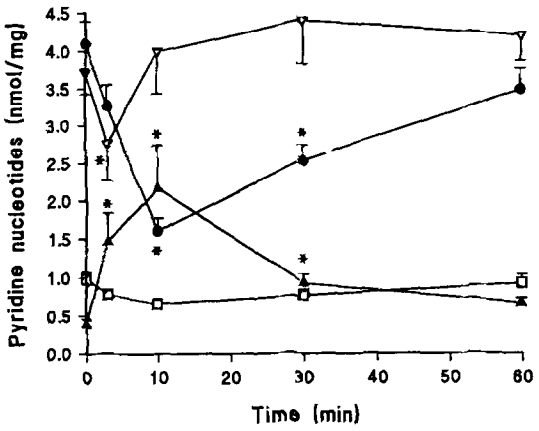


Fig. 3. The effects of 2,3-diOMe-1,4-NQ on cellular pyridine nucleotides. Hepatocytes were exposed to 2,3-diOMe-1,4-NQ (50 μM) and at the times indicated intracellular (●) NAD^+, (□) NADH, (▲) $NADP^+$, and (▽) NADPH were determined as in Materials and Methods. Samples represent mean ± SEM of three separate incubations. *Significantly different ($P < 0.05$) from the control.

DISCUSSION

The possibility of pyridine nucleotide interconversion represented a novel cellular response to oxidative stress and was originally proposed as a mechanism whereby intracellular NAD(H) may be interconverted to NADP(H) in an attempt to combat the effects of quinone-induced oxidative stress [2, 7]. Recently, in hepatocytes exposed to tBH an early increase in $NADP^+$ was observed, accompanied by a depletion of NAD^+ which was not prevented by 3-aminobenzamide [8]. This observation led to the suggestion of an interconversion of NAD(H) to $NADP^+$, due in part to the oxidation of NADPH to $NADP^+$. The results of the present study suggest that the early increase in the level of $NADP^+$ is due to oxidation of NADPH and that the depletion of NAD^+ is the result of hydrolysis, mediated either by poly(ADP-ribose)polymerase or a related enzyme.

Table 2. The effects of poly(ADP-ribose)polymerase inhibitors on the rate of NAD^+ depletion (pmol/min) in rat hepatocytes

	poly(ADP-ribose)polymerase inhibitors			
	Alone	nico (10 mM)	3-ab (20 mM)	thp (7.5 mM)
2,3-diOme (50 μM)	113 ± 19	83 ± 20*	82 ± 21*	84 ± 20
tBH (500 μM)	129 ± 21	92 ± 21*	72 ± 17*	61 ± 23*

Hepatocytes were incubated either alone or in the presence of nicotinamide: 10 mM (nico), 3-aminobenzamide: 20 mM (3-ab) or theophylline: 7.5 mM(thp). After 1 hr the cells were exposed to either 2,3-diOMe-1,4-NQ (2,3-diOme) or tBH for 15 min and intracellular NAD^+ was determined as described in Materials and Methods. Values represent the mean ± SEM of at least three separate incubations.

* Significantly different ($P < 0.05$) from the control.

Table 3. The effect of poly(ADP-ribose)polymerase inhibitors on 2,3-diOMe1,4-NQ and tBH-induced NAD^+ depletion (% control) in rat hepatocytes

	Poly(ADP-ribose)polymerase inhibitors			
	-inhib	nico	3-ab	thp
2,3-diOMe (50 μM)	45 ± 7	25 ± 6*	28 ± 7*	35 ± 9
tBH (500 μM)	58 ± 5	28 ± 7*	25 ± 6*	33 ± 7*

Hepatocytes were incubated either in the absence (-inhib) or in the presence of nicotinamide: 10 mM (nico), 3-aminobenzamide: 20 mM (3-ab) or theophylline: 7.5 mM (thp). After 1 hr the cells were exposed to either 2,3-diOMe-1,4-NQ (2,3-diOMe), tBH for 15 min and intracellular NAD^+ was determined as described in Materials and Methods. Values represent the mean ± SEM of at least three separate incubations.

* Significantly different ($P < 0.05$) from the cells incubated in the absence of the inhibitors.

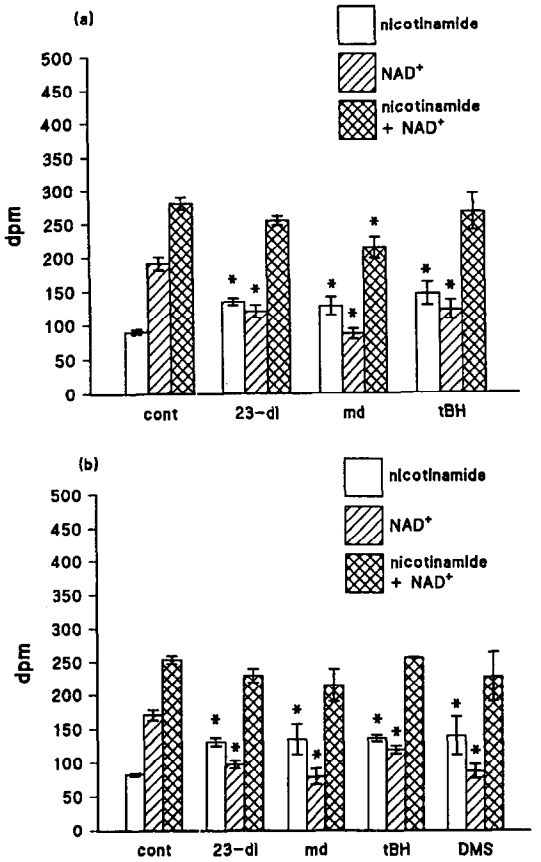


Fig. 4. The effects of quinones and tBH on the hydrolysis of NAD^+. Hepatocytes were exposed to either tBH (tBH) 500 μM, 2,3-diOMe-1,4-NQ (23-di) 50 μM, menadione (md) 50 μM, or dimethyl sulphate (DMS): 800 μM. After either (a) 15 min, or (b) 30 min NAD^+ depletion and nicotinamide formation were determined as described in Materials and Methods. Values represent the mean ± SEM of three separate incubations. *Significantly different ($P < 0.05$) from the control.

Stubberfield and others have proposed that the early increase in $NADP^+$ may be the result of the activation of an NAD^+ kinase, which converts NAD^+ to $NADP^+$ [2, 19, 20]. Although an increase in NAD^+ phosphorylation cannot be ruled out, an increase in $NADP^+$ is more likely to result from the activation of NADPH dependent reductases, which generate $NADP^+$ during the metabolism of quinones and tBH [4, 21]. By measuring intracellular pyridine nucleotides at a very early time point (3 min) after exposure to 2,3-diOMe-1,4-NQ (50 μM), a simultaneous decrease in the level of NADPH and an increase in $NADP^+$ with no change in total NADP(H) was observed (Fig. 3). Although these early changes in NADP(H) were consistent with the oxidation of NADPH, they were not detected in our earlier studies when the earliest time point was 10 min [2, 7]. Between 3 and 10 min, there was an increase in the level of $NADP^+$ and an increase in total NADP(H) (Fig. 3), which coincided with the extensive oxidation of NADPH and may reflect a greater recovery of NADP(H) when the nucleotide is in the oxidized ($NADP^+$) form. After 10 min incubation, there was an increase in the level of NADPH and NADP(H), while the level of $NADP^+$ decreased to control values (Table 1). The increase in NADPH may be the result of the activation of the hexose monophosphate shunt which reduces $NADP^+$ to NADPH. The shunt is activated by a decrease in the ratio of NADPH/$NADP^+$ [22], similar to that observed in hepatocytes to 2,3-diOMe-1,4-NQ after 3 min. Following the activation of the shunt, the significant proportion of the cellular NADPH may remain outside the mitochondrial compartment, so facilitating the recovery of NADPH and contributing to the increase in total NADP(H). After 60 min, the decrease in NADPH and NADP(H) may reflect the movement of NADPH into the mitochondria. In hepatocytes exposed to tBH (250–750 μM), less oxidation of NADPH was observed, consequently no increase in total NADP(H) was detected (Fig. 2).

The earlier evidence for nucleotide interconversion was supported by the observation that the depletion of NAD^+ occurred in the presence of 3-amino-benzamide and therefore may not be the result of

hydrolysis [7, 8]. In the present study, when hepatocytes were preincubated for one hour with inhibitors of poly(ADP-ribose)polymerase a reduction in both the rate and percentage of cellular NAD^+ depleted by 2,3-diOMe-1,4-NQ and tBH was observed (Tables 2 and 3). A 1 hr preincubation was necessary to allow the inhibitor to accumulate in the cell [23]. These results suggest the involvement of either poly(ADP-ribose)polymerase or a related enzyme in the depletion of NAD^+. In previous studies [7, 8, 17], the failure to inhibit the depletion of NAD^+ may be due in part to the very short preincubation of the inhibitors employed.

Further support of the involvement of poly(ADP-ribose)polymerase resulted from the studies with radiolabelled nicotinamide, when a simultaneous decrease in the level of NAD^+ and an increase in nicotinamide was observed following exposure to either 2,3-diOMe-1,4-NQ or tBH (Fig. 4) [15, 24]. Extensive NAD^+ hydrolysis was observed in cells exposed to both 2,3-diOMe-1,4-NQ and tBH, whereas an increase in total NADP(H) was observed only in cells exposed to 2,3-diOMe-1,4-NQ (Table 1). These observations dissociate at least under these conditions the depletion of NAD^+ and the increase in NADP(H).

Redox cycling quinones and hydroperoxides have been widely reported to cause DNA damage [25, 26]. Activation of poly(ADP-ribose)polymerase and subsequent depletion of NAD^+ are associated with DNA repair [27]. In contrast to earlier studies [2, 7, 17] these results suggest that the depletion of NAD^+ observed in hepatocytes during oxidative stress is the result of hydrolysis possibly mediated by the activation of poly(ADP-ribose)polymerase rather than an interconversion of NAD^+ to $NADP^+$.

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