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NAD^+ DEPLETION AND CYTOTOXICITY IN ISOLATED HEPATOCYTES

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Abstract—Activation of poly(ADP-ribose)polymerase by DNA damaging agents causes a depletion of intracellular NAD^+ and subsequent lowering of ATP pools, which if extensive may lead to cell death. We have studied the cytotoxicity to isolated hepatocytes of dimethyl sulphate, a direct-acting carcinogen and mutagen, hydrogen peroxide, generated by glucose/glucose oxidase, and menadione (2-methyl-1,4-naphthoquinone) in relation to their effects on intracellular NAD^+ and ATP levels. Both dimethyl sulphate and glucose/glucose oxidase caused a depletion of NAD^+, which was apparently due to an activation of poly(ADP-ribose)polymerase as it was prevented by inhibitors of the polymerase, i.e. 3-aminobenzamide and nicotinamide. This protection of intracellular NAD^+ was accompanied by a prevention of the cytotoxicity of both dimethyl sulphate and glucose/glucose oxidase, while it did not alter the decrease in intracellular ATP they induced. This apparent dissociation of effects on ATP from NAD^+ does not support the suggestion that activation of poly(ADP-ribose)polymerase leads to a decrease in cellular ATP as a consequence of NAD^+ depletion. Menadione also caused a depletion of NAD^+ which preceded cytotoxicity, but in contrast to dimethyl sulphate and H_2O_2 this depletion did not involve poly(ADP-ribose)polymerase as it was not prevented by inhibitors of the enzyme. Our results also indicate that the cytotoxicity of menadione is not mediated by H_2O_2 alone. Marked depletion of intracellular NAD^+ prior to toxicity and a protection against toxicity associated with maintenance of NAD^+ suggest a possible role for the maintenance of intracellular NAD^+ in cellular integrity.

A number of drugs and chemicals, including adriamycin, menadione (2-methyl-1,4-naphthoquinone) and paraquat, are believed to cause toxicity by oxidative stress [1, 2]. The cytoxicity of menadione is preceded by a depletion of intracellular reduced glutathione and an increase in oxidized glutathione, followed by a depletion of protein sulphydryl groups and the appearance of blebs on the plasma membrane [3–5]. This oxidative stress results in the oxidation of critical sulphydryl groups in ATP-dependent Ca^{2+} translocases causing their inactivation and a disturbance of intracellular Ca^{2+} homeostasis; in particular a marked and persistent elevation of cytosolic free Ca^{2+} [6, 7]. The mechanism by which this perturbation of Ca^{2+} homeostasis causes cell death is unclear, but it may involve the activation of Ca^{2+} dependent proteases [8] and/or phospholipases [9].

The initial step in the metabolic activation of menadione is most probably its one electron reduction to the semiquinone radical, which will react with molecular oxygen forming O_2^-, regenerating menadione and completing a redox cycle [1, 3]. Dismutation of O_2^-, either spontaneously or enzymically by superoxide dismutase, produces H_2O_2, which can be metabolized to water by glutathione peroxidase with the oxidation of GSH to GSSG. It has been suggested that the toxicity of menadione may in part be due to H_2O_2 or other active oxygen species [3].

Recently Schrauffstatter *et al.* have studied the cytotoxicity of H_2O_2 to the $P388D_1$ murine macrophage-like cell line [10]. 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^+ appears to be due to H_2O_2-induced DNA strand breaks causing an activation of poly(ADP-ribose)polymerase (EC 2.4.2.30) [11, 12]. Polymerase activation and NAD^+ loss are observed following exposure to a number of DNA-damaging agents, including alkylating agents and ionizing radiation [13]. ADP-ribosylation is a form of post-translational protein modification, involving cleavage of the β-*N*-glycosidic link of NAD^+ to obtain the ADP-ribose group, which is added to specific amino acid residues, with the subsequent release of nicotinamide [14]. The nuclear poly(ADP-ribose)polymerase also catalyzes polymerisation by addition of further ADP-ribose moieties and in response to single strand breaks stimulates DNA repair through modulation of DNA ligase II activity [15]. 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 [12].

Low concentrations of menadione (25 μM) also cause DNA strand breaks [16]. We have therefore investigated a possible role for poly(ADP-

ribose)polymerase in the toxicity of menadione in hepatocytes and compared its effects to those of H_2O_2, generated by glucose/glucose oxidase, and dimethyl sulphate. Dimethyl sulphate is a DNA alkylating agent, which in a wide range of cell types, induces single strand breaks and activates poly(ADP-ribose)polymerase [13]. The effects of this activation, i.e. DNA ligation and NAD^+ depletion, can be prevented by inhibitors of poly(ADP-ribose)-polymerase.

Menadione, H_2O_2 and dimethyl sulphate all produced a rapid, concentration-dependent 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 and H_2O_2, but not with menadione. The depletion of NAD^+ caused by menadione was possibly due to a NAD^+-glycohydrolase other than poly(ADP-ribose)-polymerase.

MATERIALS AND METHODS

Collagenase, HEPES, 3-aminobenzamide, firefly lantern extract, thiazolyl blue, phenazine ethosulphate, alcohol dehydrogenase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase and all nucleotides were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). Dimethyl sulphate, 2-methyl-1,4-naphthoquinone and tributylamine were from Aldrich Chemical Co. Ltd (Gillingham, Dorset, U.K.), the MEM amino acid mixture from Flow Laboratories plc (Aylesbury, Bucks, U.K.), and [^{3}H]-leucine from Amersham International (Rickmansworth, Herts, U.K.). All remaining reagents were purchased from BDH Ltd (Poole, Dorset, U.K.).

Cell isolation and incubation. Hepatocytes were prepared from male ICI Wistar rats (ICI plc, Alderley Park, Macclesfield, Cheshire) using Sigma type IV collagenase [17]. Perfusion yielded an initial cell viability of 90–95% and following a 30 min pre-incubation, viabilities of 80–85% were obtained immediately before treatment. The cells were incubated in rotating, 25 ml round bottomed flasks at a concentration of 10^6 cells/ml, in a balanced salt solution containing 135 mM NaCl, 4.5 KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 1.5 mM NaH_2PO_4 and 0.1% glucose, and gassed continuously with carbogen (95% O_2/5% CO_2). For protein synthesis determination, the solution was supplemented with a fifth of the standard concentration of MEM amino acids and 1 μCi/ml [^{3}H]-leucine.

Viability was determined by trypan blue dye exclusion in a 0.2% solution.

Sample preparation. Aliquots of cell suspension (equivalent to 10^6 cells) were sedimented by centrifugation at 300 rpm and the supernatant fraction aspirated. NAD^+ was extracted by addition of 0.5 M perchloric acid (PCA) and the acid extract stored at −80° prior to analysis, with a further aliquot being stored separately for ATP determination. The protein pellet was washed three times in 2 ml 10% TCA and digested in 0.25 ml 1 M NaOH. A sample from the digest (0.15 ml) was used to determine the amount of protein synthesis that had occurred and the remainder was diluted with 0.5 M NaOH and assayed for protein content by the method of Lowry *et al.* [18].

A second aliquot of cell suspension (10^6 cells) was centrifuged and the medium aspirated. The cells were precipitated with 3 ml 99% ethanol/1% 5 M KOH, and following centrifugation the pellet was resuspended in 1.0 ml chloroform. 0.5 ml of 0.01 M KOH was added and after vigorous mixing the two phases were separated by centrifugation and the aqueous phase stored at −80° for NADH measurement.

ATP measurement. ATP levels were determined using a luciferase-linked luminescence method [19, 20]. An aliquot of the PCA-extract (10 μl) was diluted with 2 ml 33 mM $Na_2HAs_5O_4$/27 mM $MgSO_4$/3.3 mM KH_2PO_4, pH 7.4 and the reaction was initiated by the addition of 0.1 ml firefly lantern extract. The luminescence was measured using a photon detection system Mk II, incorporating a 9863B98/350 red-sensitive tube (Thorn EMI Ltd, Ruislip, Middlesex, U.K.), forced-air cooled to −25° and calibrated with a range of concentrations of ATP (0–40 μM).

Nucleotide determinations. NAD^+ was measured using a spectrophotometric thiazolyl blue/phenazine ethosulphate-linked recycling assay [21]. The assay contained 120 mM ethanol and 12.5 units/ml alcohol dehydrogenase in 0.1 M potassium phosphate pH 7.6 with thiazolyl blue and phenazine ethosulphate concentrations of 50 μM and 200 μM respectively. Prior to analysis, the acid extract was buffered to pH 7.4 with 3M KOH. The rate of increase in absorbance at 570 mm was proportional to the amount of nucleotide present.

NADH was separated by isocratic elution with 0.2 M ammonium phosphate/17.87% methanol/0.13% tributylamine pH 6.0 from a C18 reverse-phase HPLC column [22]. The eluent was monitored continuously at 340 nm and the peak area calculated by integration.

RESULTS

NAD^+ depletion and viability

In agreement with previous studies [3, 4, 23], menadione caused a concentration-dependent toxicity to hepatocytes (Fig. 1a), although its toxicity was slightly greater than in earlier studies possibly due to differences in the incubation media. Menadione (20–200 μM) caused a concentration-dependent decrease in intracellular NAD^+ levels (Fig. 1b). A low non-toxic concentration of menadione (20 μM) produced a transient fall in NAD^+ levels, whilst concentrations of 50 μM and above caused a very marked initial decrease (Fig. 1b). After 30 min incubation, a partial recovery of NAD^+ levels was observed at the non-toxic 50 μM concentration but not with the toxic 100 and 200 μM concentrations (Fig. 1b). At these toxic concentrations, NAD^+ levels continued to fall throughout the incubation prior to cell death (compare Figs 1a and b).

Dimethyl sulphate caused a concentration-dependent toxicity to hepatocytes (results not shown). High concentrations (500 μM) caused a time-depen-

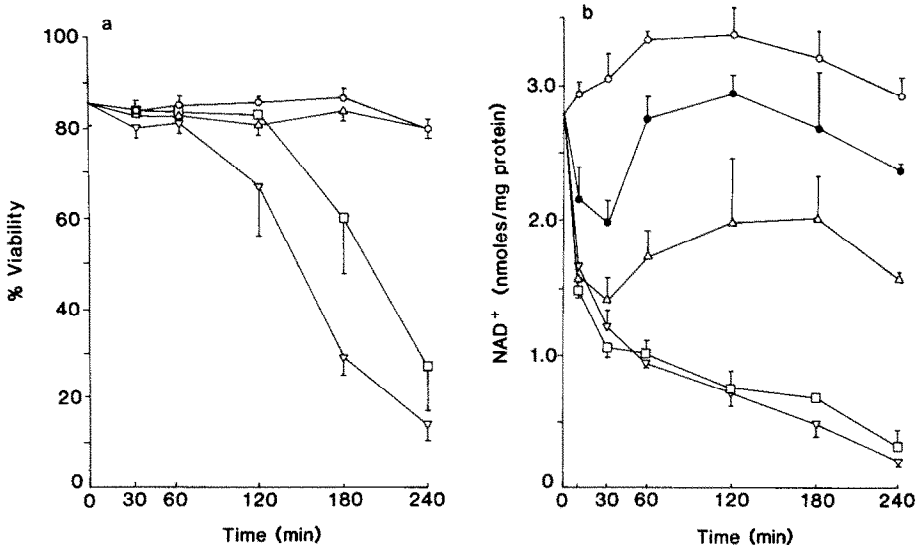

Fig. 1. Effects of menadione on (a) cell viability and (b) NAD^+ depletion in isolated hepatocytes. Cells were incubated either alone (○—○) or with the following concentrations of menadione: 20 μM (●—●), 50 μM (△—△), 100 μM (□—□) or 200 μM (▽—▽). NAD^+ was measured as described in Materials and Methods and viability was assessed by trypan blue dye exclusion. Menadione (20 μM) had no effect on viability. Results represent the mean ± SEM of at least three determinations (average protein = 1.75 mg/10^6 cells).

dent cytotoxicity which was partially prevented by low (2.5 mM) and almost completely prevented by high (20 mM) concentrations of 3-aminobenzamide (Fig. 2a). The toxicity was preceded by a depletion of NAD^+ (to 5% of control values within 180 min (Fig. 2b)) and the effects of 3-aminobenzamide on NAD^+ paralleled those on toxicity with the loss being partly or more completely prevented by low or high concentrations of 3-aminobenzamide respectively (Fig. 2b).

Spragg and co-workers [24] used both H_2O_2 and glucose/glucose oxidase to stimulate DNA damage and NAD^+ depletion in P388D_1 cells. In the present study, it was felt that the glucose/glucose oxidase couple should resemble more closely the production of H_2O_2 from the redox cycling of menadione than would the addition of a single bolus of H_2O_2. Addition of glucose oxidase (5 units/ml) to the medium, with 0.1% glucose, caused a time-dependent cytotoxicity which was partially prevented by 3-aminobenzamide (20 mM) (Fig. 3a). This concentration of glucose/glucose oxidase also caused a

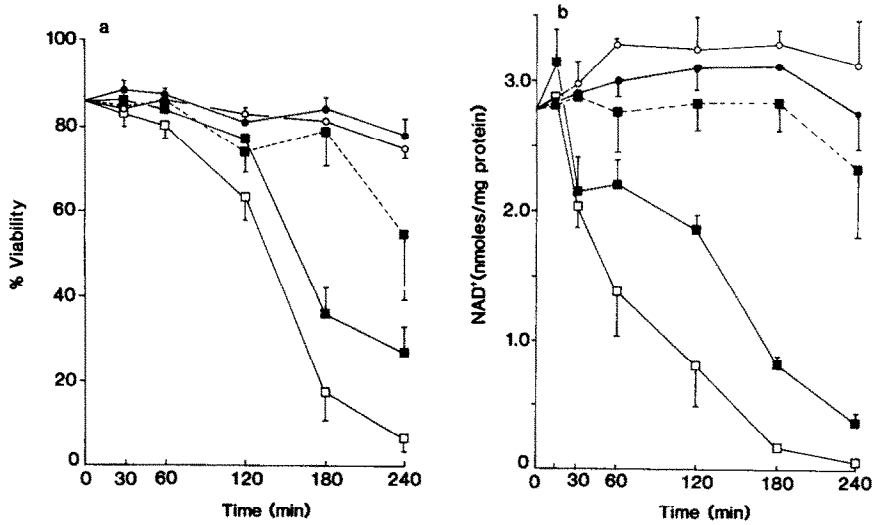

Fig. 2. Effects of 3-aminobenzamide on (a) cell viability and (b) NAD^+ depletion produced by dimethyl sulphate. Control hepatocytes were incubated in the presence (●—●) or absence (○—○) of 20 mM 3-aminobenzamide. Hepatocytes were also incubated with 500 μM dimethyl sulphate alone (□—□), or in the presence of either 2.5 mM (■—■) or 20 mM 3-aminobenzamide (■---■). Viability was measured by trypan blue dye exclusion and NAD^+ as described in Materials and Methods (average protein = 1.78 mg/10^6 cells). 3-Aminobenzamide (2.5 mM) had no effect on control viability or NAD^+ levels.

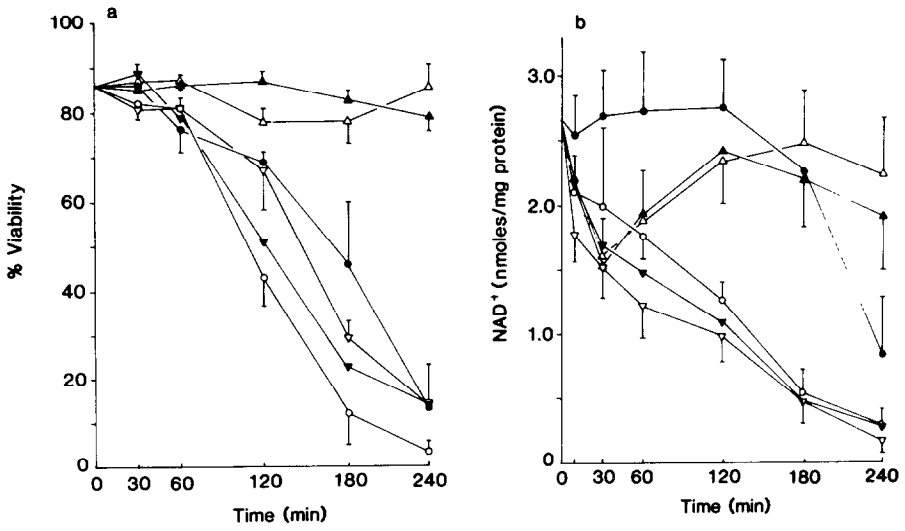

Fig. 3. Effects of 3-aminobenzamide (20 mM) on (a) cell viability and (b) NAD^+ depletion associated with glucose/glucose oxidase (○,●), and menadione, 50 μM (△,▲) and 200 μM (▽, ▼). The open and closed symbols represent the incubations without and with 3-aminobenzamide respectively. Viability was assessed by trypan blue dye exclusion and NAD^+ was measured as described in Materials and Methods (the average protein content was 1.88 mg/10^6 cells). Results represent the Mean ± SE of at least three determinations.

marked depletion of intracellular NAD^+. This depletion was extensively prevented up to 180 min by the higher 3-aminobenzamide concentration (20 mM) (Fig. 3b).

3-Aminobenzamide failed to effect the changes in NAD^+ levels produced by either 50 or 200 μM menadione (Fig. 3b), and it may have caused a slight potentiation of the cell death produced by the higher concentration of menadione (Fig. 3a).

Other inhibitors of poly(ADP-ribose)polymerase, nicotinamide and theophylline, also protected against the cytotoxicity of dimethyl sulphate, but failed to alter the cytotoxicity of menadione (Table 1).

Effects on ATP

It has been suggested that depletion of ATP is a consequence of the fall in NAD^+ following H_2O_2 exposure [25–27], and hence, by preventing the NAD^+ loss, poly(ADP-ribose)polymerase inhibitors will prevent the ATP loss. Both dimethyl sulphate and glucose/glucose oxidase caused a time-dependent depletion of ATP, which was not prevented by 3-aminobenzamide (Fig. 4b). The lower concentration of menadione (50 μM) caused only a small and short-lived reduction of ATP levels (Fig. 4a) while 200 μM caused an extensive depletion of intracellular ATP levels (Fig. 4a) which preceded cell death, but followed the loss of NAD^+ (Fig. 1).

Table 1. Effects of poly(ADP-ribose)polymerase inhibitors on toxicity and NAD^+-depletion produced by dimethyl sulphate and menadione

Incubation conditions	% Cell viability*	nmoles NAD^+/ mg protein†‡
Dimethyl sulphate	17 ± 7	0.15 ± 0.04
Dimethyl sulphate + 2.5 mM 3-aminobenzamide	36 ± 5	0.82 ± 0.07
Dimethyl sulphate + 20 mM 3-aminobenzamide	79 ± 8	2.78 ± 0.23
Dimethyl sulphate + 10 mM nicotinamide	46 ± 7	0.78 ± 0.20
Dimethyl sulphate + 10 mM theophylline	32 ± 8	0.99 ± 0.29
Menadione	28 ± 5	0.48 ± 0.12
Menadione + 20 mM 3-aminobenzamide	23 ± 13	0.49 ± 0.19
Menadione + 10 mM nicotinamide	34 ± 7	0.52 ± 0.20
Menadione + 10 mM theophylline	31 ± 8	0.38 ± 0.15

Hepatocytes were incubated with either dimethyl sulphate (500 μM) or menadione (200 μM) in the presence of various inhibitors of poly(ADP-ribose)polymerase. NAD^+ content and cell viability were determined as described in the Materials and Methods and the figures presented are the mean ± SE from three determinations obtained after 180 min incubation.

* Control viability was 81 ± 2%.

† Control NAD^+ level was 3.26 ± 0.09 nmoles/mg protein.

‡ Average protein content was 1.74 mg/10^6 cells.

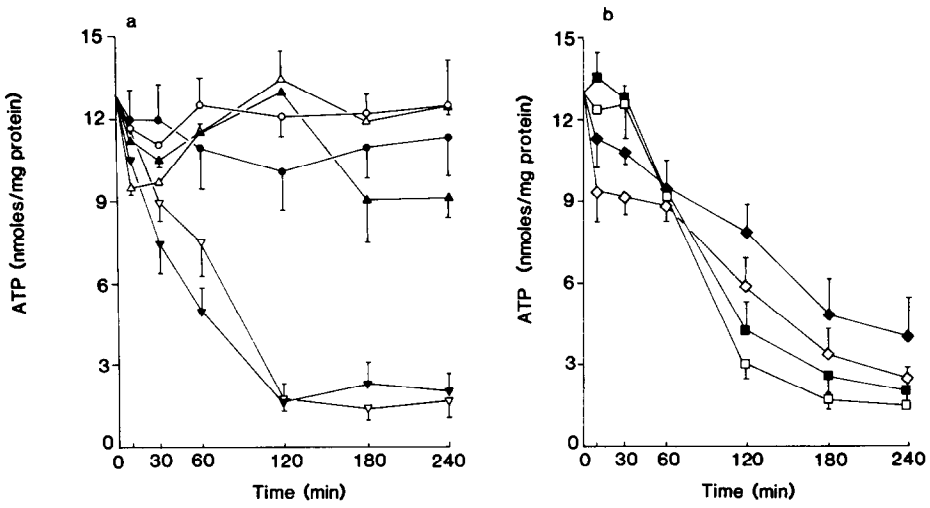

Fig. 4. Effects of menadione, glucose/glucose oxidase and dimethyl sulphate on intracellular ATP levels in the presence or absence of 3-aminobenzamide (20 mM). Hepatocytes were incubated either alone (○,●), with menadione (50 μM) (△,▲), or 200 μM (▽, ▼), with glucose/glucose oxidase (5 units/ml) (◇, ◆), or with dimethyl sulphate (500 μM) (□,■). The open and closed symbols represent the incubations without and with 3-aminobenzamide respectively. ATP levels were determined in the perchloric acid extract of the hepatocytes using the firefly luciferase-linked luminescence method as described in Materials and Methods. The average protein content was 1.65 mg/10^6 cells. The results represent the mean ± SEM of at least three determinations.

3-Aminobenzamide (20 mM) had little or no effect on intracellular levels of ATP in control hepatocytes or in presence of menadione (50 and 200 μM) (Fig. 4a).

Analysis of NADH levels

In order to investigate whether the loss of intracellular NAD^+ caused by menadione (Fig. 1) was due to an alteration in the redox state in favour of NADH, the cellular content of NADH was determined (Fig. 5). Control levels of NADH remained almost constant throughout the incubation period (Fig. 5). In the presence of menadione (200 μM), a rapid decline in NADH was observed over the first 30 min followed by a slower fall over the rest of the incubation period (Fig. 5). When the results were expressed as the ratio of NAD^+:NADH, they showed a shift in favour of the oxidized state (Fig. 5).

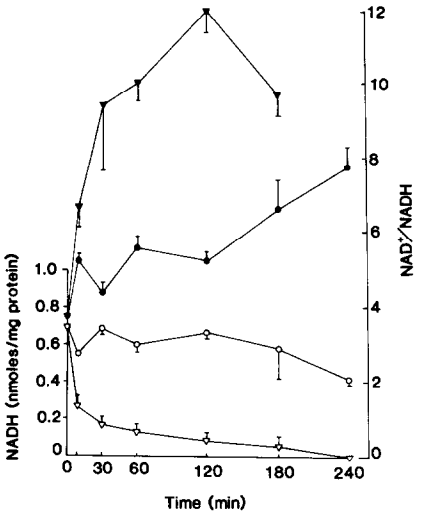

Fig. 5. The effects of menadione on NADH levels and the NAD^+/NADH ratio in isolated hepatocytes. Hepatocytes were incubated either alone (○,●) or in the presence of menadione (200 μM) (▽, ▼). Open and closed symbols represent the NADH levels and NAD^+/NADH ratios respectively. NADH and NAD^+ were measured by the methods described in Materials and Methods. The average protein content = 1.71 mg/10^6 cells.

DISCUSSION

Poly(ADP-ribose)polymerase activation and inhibition

The activation of poly(ADP-ribose)polymerase, as a consequence of DNA single strand breaks, may play an important role in the regulation of the response of the cell to DNA damage [26–28]. Low levels of damage activate the polymerase causing a moderate loss of NAD^+ and an increased rate of DNA repair and ligation [29]. When DNA is extensively damaged, activation of the polymerase causes a greater hydrolysis of NAD^+, reducing levels to a point at which the cell cannot survive [26, 27]. In multicellular organisms, the rapid death of the cell due to NAD^+ loss may be preferable if it avoids the risk of replication following extensive DNA repair with a high level of infidelity.

The protection afforded by the poly(ADP-ribose)polymerase inhibitor, 3-aminobenzamide, against the cytotoxicity and NAD^+ depletion induced by both dimethyl sulphate and glucose/glucose oxidase (Fig. 3) supported the involvement of the polymerase in the cytotoxicities of these agents. This was further supported by the protective effects of nicotinamide and theophylline (Table 1), both

Table 2. Effects of 3-aminobenzamide on the inhibition of protein synthesis by dimethyl sulphate, glucose/glucose oxidase and menadione

Incubation	Protein synthesis dpm incorporated/mg protein/hour Control	3-Aminobenzamide (2.5 mM)	3-Aminobenzamide (20 mM)
Control	28472 ± 1693	28361 ± 1915	11516 ± 2969
Dimethyl sulphate (500 μM)	1415 ± 278	833 ± 56	916 ± 527
Glucose/glucose oxidase (5 units/ml)	416 ± 139	500 ± 139	389 ± 167
Menadione (50 μM)	6327 ± 1360	7770 ± 666	2165 ± 1082
Menadione (200 μM)	833 ± 167	1388 ± 747	833 ± 416

Protein synthesis was determined in hepatocytes in the presence or absence of 3-aminobenzamide (2.5 or 20 mM) by measuring the rate of incorporation of [^{3}H]-leucine into acid-precipitable material as described in Materials and Methods. The figures represent the mean ± SE from three experiments.

known inhibitors of poly(ADP-ribose)polymerase [13]. In contrast to these results, 3-aminobenzamide (and nicotinamide and theophylline (Table 1)) had no effect on either the loss of viability caused by menadione (200 μM) (Fig. 3) or on its depletion of NAD^+ (50 and 200 μM) (Fig. 3). This lack of alteration in NAD^+ loss in the presence of the polymerase inhibitors implied that there was no activation of poly(ADP-ribose)polymerase associated with the DNA damage produced by menadione (25 μM) [16] in intact hepatocytes. Whereas results with H_2O_2 demonstrate activation of the poly(ADP-ribose)polymerase [11], activation is not common to all types of DNA damage [13]. It is possible that the damage induced by menadione is not caused by H_2O_2 produced through redox cycling, but by DNA-metabolite adducts [16], which do not activate the polymerase. Alternatively menadione may arylate [30] essential thiol groups in poly(ADP-ribose)-polymerase causing its inactivation [14].

ATP depletion

Previous studies with inhibitors of poly(ADP-ribose)polymerase showed that the fall in ATP levels which followed NAD^+ depletion was averted when NAD^+ levels were maintained [11, 27]. In the present studies, 3-aminobenzamide caused a small, but not significant, protection of ATP levels following treatments with either dimethyl sulphate or glucose/glucose oxidase (Fig. 4). The loss of ATP is believed to be a result of the lack of NAD^+ available for glycolysis and/or oxidative phosphorylation [26] and an activation of the hexose monophosphate shunt [24]. The reason for the difference between this study and others may relate to differences in the relative importance of the two routes of ATP synthesis between hepatocytes and the cell types used in other studies (L1210, P388D$_1$, and human lymphocytes).

Interestingly, the results appear to dissociate maintenance of ATP from cell viability, since with the cells treated with dimethyl sulphate or glucose/glucose oxidase, 3-aminobenzamide prolonged viability without maintaining ATP (Fig. 4). However, the slight protection of ATP levels by 3-aminobenzamide (Fig. 4) may be sufficient to raise them above a critical "threshold" level below which the cell dies.

NAD^+ levels and cell viability

Whilst exposure to 3-aminobenzamide protected the hepatocytes against the cytotoxicity of dimethyl sulphate as assessed by trypan blue dye exclusion (Fig. 2), it is unclear whether this was due directly to the preservation of NAD^+ levels. In the presence of 3-aminobenzamide, the loss of NAD^+ accompanied rather than preceded cell death indicating that levels of NAD^+ alone cannot determine viability. The higher intracellular levels of NAD^+ may allow other processes to continue for a longer period of time, or other consequences of the treatment such as DNA damage, may ultimately cause cell death.

In marked contrast to the protective effect of 3-aminobenzamide on the cytotoxicities of dimethyl sulphate or glucose/glucose oxidase as assessed by dye exclusion (Figs 2 and 3), no protection was observed using protein synthesis as an alternative marker of cell viability (Table 2). This lack of protection may have been due to the inability of 3-aminobenzamide to protect against the fall in intracellular ATP (Fig. 4), which is required for protein synthesis. It was also noted that higher concentrations of 3-aminobenzamide (20 mM) alone caused a marked inhibition of protein synthesis (Table 2). No protection of the toxicity of menadione was observed using protein synthesis as a marker of toxicity (Table 2).

The poor specificity of the polymerase inhibitors and their use at concentrations apparently well above those required for enzyme inhibition has been highlighted [31]. Theophylline inhibits cyclic-AMP phosphodiesterase and benzamide and its derivatives inhibit phosphodiesterase, carboxypeptidase A, chymotrypsin and nicotinamide *N*-methyltransferase [32]. Nicotinamide acts as a substrate for many enzymes, e.g. *N*-methyltransferase and phosphoribosyltransferase and therefore has the potential to alter the metabolism of the cell in many ways. Other possibly important aspects of polymerase inhibition are the roles of polyADP-ribosylation in the modulation of other enzymes, e.g. topoisomerases I and II [33], DNA polymerase α and β [34], RNA polymerase II [35], ribonuclease [36] and Ca^{2+}, Mg^{2+} endonuclease [37]. Alterations in these activities may have profound effects on DNA and RNA synthesis.

Our results did not show any differences in the response of the cells to the different concentrations of 3-aminobenzamide only that the higher concentration extended the period of protection against dimethyl sulphate (Fig. 2). The metabolism of 3-aminobenzamide has not been reported, but it is possible that the higher concentration simply ensures a longer period of polymerase inhibition in the more metabolically active hepatocytes used in these studies.

Mechanism of NAD(H) loss following menadione treatment

Menadione (200 μM) caused a major loss of NAD^+ (Fig. 1) and NADH (Fig. 5) from the cell which preceded cell death (Fig. 1). The absence of a protective effect of 3-aminobenzamide on the NAD^+ and NADH loss caused by menadione (Figs. 3 and 5) suggested that the depletion of NAD^+ was proceeding by a route other than activation of poly(ADP-ribose) polymerase.

Analysis of the incubation medium showed no leakage of NAD(H) from the cell prior to loss of integrity of the cell membrane (data not shown), and hence the loss of NAD(H) must be due to further metabolism. In eukaryotic cells, the major degradative route for NAD^+ is via hydrolysis to nicotinamide and ADP-ribose, with the bulk of the breakdown occurring in the nucleus [38]. In addition to the NAD^+-glycohydrolase activity of poly(ADP-ribose) polymerase, mitochondrial and plasma membrane NAD^+-glycohydrolase activities have been reported [39]. Richter *et al.* have reported an increase in mono(ADP-ribosylation) of rat liver mitochondrial proteins [40] associated with hydroperoxide-induced oxidation and hydrolysis of pyridine nucleotides and the release of Ca^{2+} from mitochondria. However, in control hepatocytes, approximately 80% of NAD is extramitochondrial [41] and since exchange of NAD^+ across mitochondrial membranes is slow (1%/min [42]) it seems likely that any rapid loss, such as observed here with menadione (Fig. 1), would occur outside the mitochondria. Furthermore, mitochondrial NAD^+-glycohydrolase is inhibited by nicotinamide [43] and 3-aminobenzamide [43, 44] and our results showed no protection of menadione-induced NAD^+ depletion by these agents (Table 1). It would therefore appear that depletion of NAD^+ by menadione is probably due to a cytosolic NAD^+-glycohydrolase; however, other possibilities such as an interconversion of nucleotides may also be responsible.

In summary, menadione, dimethyl sulphate and glucose/glucose oxidase produced a rapid oxidation and loss of NAD(H) which preceded the fall in ATP levels and cell death. NAD^+ depletion and cytotoxicity induced by dimethyl sulphate and glucose/glucose oxidase but not menadione were prevented by poly(ADP-ribose)polymerase inhibitors indicating that the polymerase was not responsible for the NAD(H) loss associated with menadione treatment. A marked depletion of intracellular NAD^+ prior to toxicity suggested a key role for the maintenance of intracellular NAD^+ in cellular integrity.

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