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METABOLISM OF PYRIDINE NUCLEOTIDES IN CULTURED RAT HEPATOCYTES INTOXICATED WITH *tert*-BUTYL HYDROPEROXIDE

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Abstract—The alterations in the metabolism of pyridine nucleotides, as well as the role such changes play in the genesis of lethal cell injury, were explored in cultured rat hepatocytes intoxicated with *tert*-butyl hydroperoxide (TBHP). The loss of NADPH, NADH, and NAD equalled the increase in NADP, with little if any change in the total content of pyridine nucleotides. Identical alterations occurred in the presence of *N*,*N*′-diphenyl-*p*-phenylenediamine, an antioxidant that prevented the death of the cells. Inhibition of glutathione reductase by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) reduced the extent of the increase in NADP and the decrease in NADPH. At the same time, BCNU increased the cell killing. Depletion of ATP with oligomycin reduced the loss of NAD and the accumulation of NADP. Treatment of the hepatocytes with the poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide had no effect on the depletion of NAD. Thus, all of the alterations in pyridine nucleotides that accompany the exposure of cultured hepatocytes to TBHP can be dissociated from the development of lethal cell injury. The changes do suggest, however, a rapid interconversion of the respective species. The initial response reflects activation of glutathione reductase with the consequent oxidation of NADPH to NADP. The conversion of NADH to NAD and then NAD to NADP, the latter by nicotinamide adenine dinucleotide kinase, can account for the increase in NADP over that resulting from the oxidation of NADPH by glutathione reductase. Finally, there was no evidence in cultured hepatocytes treated with TBHP for changes in NAD that reflect the activation of poly(ADP-ribose) polymerase.

Changes in the redox ratio of pyridine nucleotides, as well as in their total content, heve been implicated in the genesis of irreversible cell injury with an oxidative stress. In this regard, oxidative cell injury has been attributed to the increase in the cytosolic free calcium ion concentration that occurs under oxidizing conditions [1–4]. Initially, calcium release was argued to occur from both the endoplasmic reticulum and mitochondria, the former in relation to the oxidation of glutathione and the latter to the oxidation of NADPH [5]. However, mitochondria do not normally sequester significant stores of calcium [6], and the oxidation of NADPH is not now directly implicated in the alteration in intracellular calcium homeostasis induced by an oxidative stress. Furthermore, the altered calcium homeostasis is itself an epiphenomenon that accompanies the metabolic alterations attendant on the metabolism of hydrogen peroxide by glutathione peroxidase [7].

Changes in the cellular content of NADH and NAD also occur with oxidative cell injury. The activation of poly(ADP-ribose) polymerase, a nuclear enzyme that catalyzes the transfer of ADP-ribose from NAD to protein, accompanies the induction of repair mechanisms upon the appearance of single-strand breaks in DNA. The metabolic consequences of the activation of this enzyme, namely the consumption of NAD and a consequent depletion of ATP, may be involved in the pathogenesis of oxidant-induced cell death [8, 9]. Finally, the depletion of NAD by mechanisms unrelated to the activation of poly(ADP-ribose) polymerase has been suggested to play an important role in the loss of cellular integrity in the killing of suspensions of isolated hepatocytes by hydrogen peroxide [10].

The killing of cultured hepatocytes by *tert*-butyl hydroperoxide (TBHP‡) is a useful model with which to study the mechanisms of oxidative cell injury [11–15]. This system can be manipulated in a number of specific ways that have allowed assessment of the relationship of several biochemical alterations to the genesis of irreversible injury [11–14]. In the present report, a similar approach has been taken in the analysis of the alterations in pyridine nucleotide metabolism that accompany the killing of cultured hepatocytes by TBHP.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–200 g) were obtained from the Charles River Breeding Laboratory (Wilmington, MA). All animals were fed *ad lib.* and fasted overnight prior to use. Isolated hepatocytes were prepared by collagenase (Sigma Chemical Co., St. Louis, MO) perfusion according

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‡ Abbreviations: TBHP, *tert*-butyl hydroperoxide; LDH, lactate dehydrogenase; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; DPPD, *N*,*N*′-diphenyl-*p*-phenylenediamine; 3-AB, 3-aminobenzamide; and Me_2SO, dimethyl sulfoxide.

to Seglen [15]. Yields of 2–4 × 10^8 cells/liver with 90–95% viability by trypan blue exclusion were routinely obtained. The hepatocytes were plated in 25 cm^2 flasks (Corning Glass Works, Corning, NY) at a density of 1.33 × 10^6 cells/flask in 3 mL of Williams E medium (Gibco Laboratory, Chagrin Falls, OH) containing 10 IU/mL penicillin, 10 μg/mL streptomycin, 0.05 mg/mL gentamycin, 0.02 U/mL insulin, and 10% heat-inactivated (55° for 15 min.) fetal bovine serum (Hazelton Research Products, Lenexa, KS) (complete Williams E). After incubating the cells for 2 hr at 37° in an atmosphere of 5% CO_2–95% air, the cultures were rinsed twice with a prewarmed Hepes (Sigma) buffer (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, and 2.4 mM Hepes, pH 7.4) to remove the unattached dead cells. Complete Williams E (5 mL) was replaced, and the hepatocytes were incubated for 24 hr. The cultures were then washed and incubated in Williams E minus fetal bovine serum (incomplete Williams E) with the additions indicated in the text.

Cell viability was determined by the release of lactate dehydrogenase (LDH) into the culture medium [16]. The extent of cell killing was quantified as the percentage of the total LDH present in the hepatocytes released into the medium. The total is determined by the LDH released from the cells after treatment with Triton X-100. LDH release correlated with the measurement of viability by the exclusion of trypan blue (data not shown). All experiments were performed on three separate cultures, and the data were analyzed statistically by the unpaired Student's *t*-test.

Where indicated in the text, cultures were pretreated with deferoxamine (Ciba Pharmaceutical Co., Summit, NJ) or oligomycin (Sigma) for 60 min, and with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; Bristol-Myers Oncology Division, Syracuse, NY) for 15 min, washed twice with prewarmed Hepes buffer, and placed in 5 mL of incomplete Williams E. Deferoxamine was dissolved in 0.9% NaCl and added to the cultures at a final concentration of 12 mM. Oligomycin, *N,N'*-diphenyl-*p*-phenylenediamine (DPPD; Eastman Chemical Co., Rochester, NY) and 3-aminobenzamide (3-AB; Sigma) were dissolved in dimethyl sulfoxide and added to cultures at concentrations of 0.1 μg/mL, 1 μM and 10 mM, respectively. The final concentration of Me_2SO was 0.5% in all cases; 0.5% Me_2SO was without effect on the pyridine nucleotide content and viability of control hepatocytes, and did not modify the effects of 1.0 mM TBHP on these. BCNU was dissolved in 100% ethanol and added to cultures at a final concentration of 300 μM (0.2% ethanol). Pretreatment with 0.2% ethanol alone was without effect on the pyridine nucleotide content and viability of control hepatocytes. TBHP (Sigma) was diluted with incomplete Williams E medium and added to cultures at the concentrations indicated in the text.

The content of pyridine nucleotides was measured by adaptation of the methods of Jorgensen and Rasmussen [17] and Bernofsky and Swan [18]. After the incubations indicated in the text, the cells were washed with Hepes buffer and scraped into 3 mL of 0.33 M KOH in 30% ethanol for the determination of reduced forms (alkaline extract) and into 3 mL of 3% trichloroacetic acid (TCA) for oxidized forms (acid extract). All samples were then kept on ice for 30 min. The alkaline samples were adjusted to pH 8.2 with 1.5 mL of 0.5 M KH_2PO_4 solution, and the acid samples to pH 7.4 with 0.65 mL of 0.8 M KOH and 0.2 M Tris solution. All samples were adjusted to 5 mL by H_2O and then centrifuged for 15 min at 3000 *g* to pellet precipitated protein. NADPH and NADP were measured with the use of a glucose-6-phosphate dehydrogenase–glutathione reductase system. Reduced glutathione was detected through its reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [17]. The assay contained 0.1 M Tris, 2.5 mM glucose-6-phosphate, 0.25 mM oxidized glutathione (GSSG), 0.5 mM EDTA, 0.25 mM DTNB, glucose-6-phosphate dehydrogenase and glutathione reductase. The change in absorbance was read at 412 nm. NADH and NAD were measured with the use of a thiazolyl blue and phenazine ethosulfate-linked recycling assay [10, 18]. The assay contains 200 mM ethanol, 15 U/mL alcohol dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.6, with 50 μM thiazolyl blue and 200 μM phenazine ethosulfate. The change in absorbance was read at 570 nm. The recoveries of NADPH, and NADP, NADH and NAD added to the cultures immediately after scraping the cells were about 70%, 95%, 105% and 95%, respectively.

The protein content of each culture was determined by the method of Lowry *et al.* [19].

RESULTS

Changes in NADPH, NADP, NADH, and NAD. The killing of cultured hepatocytes by TBHP is attributable to the peroxidation of cellular lipids [11]. Antioxidants, such as DPPD, prevent both the lipid peroxidation and the cell killing [11, 13, 14]. Importantly, DPPD does not affect the changes produced by TBHP in both glutathione metabolism (the loss of GSH with a parallel accumulation of GSSG) [11] and intracellular calcium homeostasis (a rise in the cytosolic free calcium ion concentration) [11, 14]. Similarly, DPPD has no effect on the metabolism of pyridine nucleotides in hepatocytes intoxicated with TBHP. This is shown by the results of the experiment detailed in Figs. 1 and 2.

Hepatocytes in culture for 24 hr were treated with 1 mM TBHP in the presence or absence of 1 μM DPPD. Within 60 min, 70% of the hepatocytes died in the absence of DPPD, whereas there was no loss of viability in its presence. Figure 1 illustrates the changes in the cellular content of NADPH (left panel) and NADP (right panel) during the first 30 min of exposure of the hepatocytes to 1 mM TBHP in the presence or absence of DPPD. The content of NADPH decreased by 65% within 5 min, and then remained at about 50% of the initial value between 15 and 30 min. DPPD had little or no effect on the rate or extent of this depletion of NADPH.

The loss of NADPH was paralleled by an increase in NADP (Fig. 1, right panel). Within 5 min of treating the cells with TBHP, the content of NADP increased 6-fold, in the presence or absence of DPPD. Between 15 and 30 min, the content of

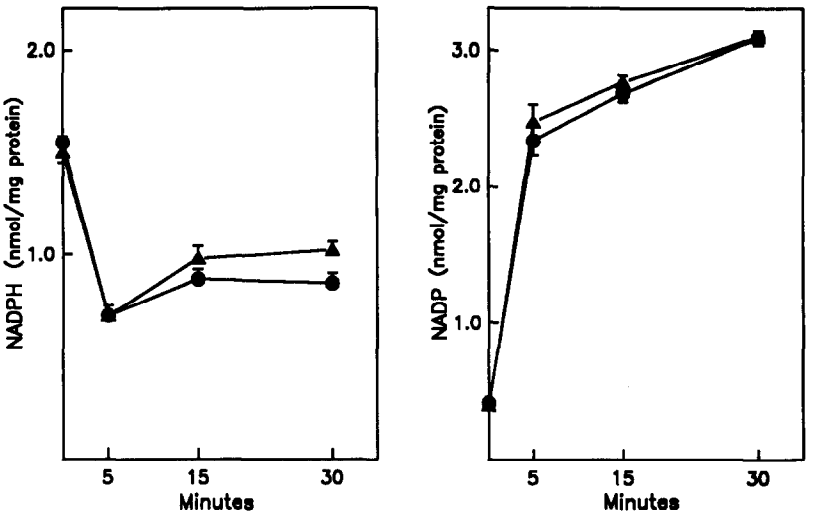

Fig. 1. Effect of *tert*-butyl hydroperoxide on the content of NADPH and NADP. Hepatocytes in culture for 24 hr were washed, placed in fresh incomplete Williams E medium (no bovine serum), and treated with 1 mM TBHP in the presence (▲) or absence (●) of 1 μM DPPD. At the times indicated, the cellular contents of NADPH (left panel) and NADP (right panel) were determined. Results are the means ± SD of determinations on three separate cultures. The difference between the NADPH content at 30 min with and without DPPD was significant at $P < 0.025$.

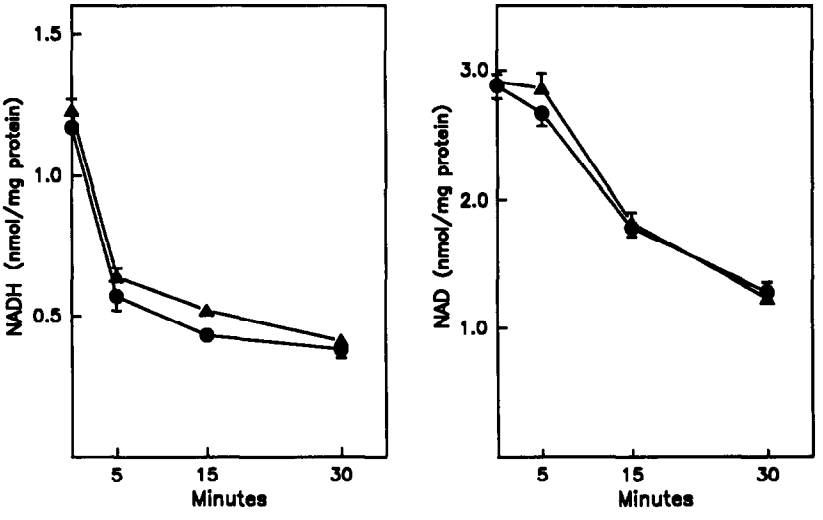

Fig. 2. Effect of *tert*-butyl hydroperoxide on the content of NADH and NAD. Hepatocytes in culture for 24 hr were washed, placed in fresh incomplete Williams E medium (no bovine serum), and treated with 1 mM TBHP in the presence (▲) or absence (●) of 1 μM DPPD. At the times indicated, the cellular contents of NADH (left panel) and NAD (right panel) were determined. Results are the means ± SD of determinations on three separate cultures.

NADP continued to increase, but at a slower rate than during the first 5 min. Again, DPPD had no effect on the changes in NADP. Within the first 30 min of exposure to TBHP, the net increase in NADP (2.68 nmol/mg protein) was 4-fold greater than the net decrease in NADPH (0.69 nmol/mg protein). Changes in the NADH and NAD content of the hepatocytes could account for the difference between the net increase in NADP and the loss of NADPH.

Figure 2 illustrates the changes in the cellular content of NADH (left panel) and NAD (right panel) during the first 30 min of exposure of the hepatocytes to 1 mM TBHP in the presence or absence of DPPD. The content of NADH decreased by 50% within 5 min. After 30 min, TBHP produced

Table 1. Pyridine nucleotide balance in rat hepatocytes intoxicated with *tert*-butyl hydroperoxide

	NADPH	NADH	NADP	NAD
		(nmol/mg protein)		
Initial values	1.55 ± 0.03	1.17 ± 0.01	0.41 ± 0.01	2.88 ± 0.01
30 min	0.86 ± 0.05	0.38 ± 0.03	3.09 ± 0.05	1.28 ± 0.08
Net change	−0.69	−0.79	+2.68	−1.60

Initial total pyridine nucleotides	6.01 nmol/mg protein
Total after 30 min	5.61 nmol/mg protein
Increase in NADP	2.68 nmol/mg protein
Loss of NADPH + NADH + NAD	3.08 nmol/mg protein

Hepatocytes were incubated with 1.0 mM TBHP for 30 min. Results are the means ± SD of determinations on three separate cultures.

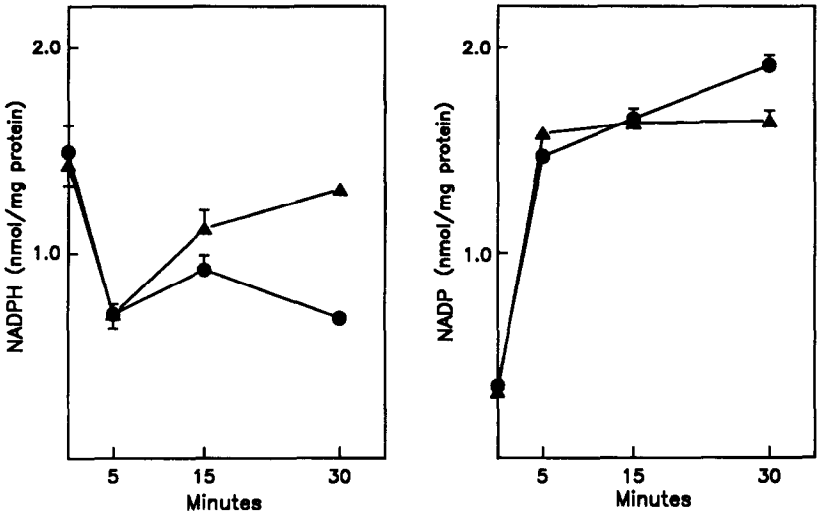

Fig. 3. Effect of pretreatment with the iron chelator deferoxamine on changes in the content of NADPH and NADP induced by *tert*-butyl hydroperoxide. Hepatocytes in culture for 24 hr were washed, placed in fresh complete Williams E medium with (▲) or without (●) 12 mM deferoxamine. After 1 hr the cultures were washed, placed in fresh incomplete Williams E medium (no bovine serum), and treated with 1 mM TBHP. At the times indicated, the cellular contents of NADPH (left panel) and NADP (right panel) were determined. Results are the means ± SD of determinations on three separate cultures. The difference between the NADPH and NADP content at 30 min with and without deferoxamine pretreatment was significant at $P < 0.001$ and $P < 0.005$, respectively.

a 70% decrease in the NADH content of the hepatocytes. Figure 2 (left panel) also indicates that DPPD had no effect on the rate or extent of this depletion of NADH.

The time course of the change in the content of NAD (Fig. 2, right panel) was somewhat different from that of the other pyridine nucleotides. There was little or no change in the content of NAD during the first 5 min of exposure to TBHP, whereas after 30 min the content of NAD had decreased by 55%. DPPD had no effect on this loss of NAD.

Table 1 summarizes the changes in NADPH, NADP, NADH, and NAD following a 30-min exposure of the cultured hepatocytes to 1 mM TBHP. There was little or no change (less than 10%) in the total content of pyridine nucleotides. In addition, the net increase in NADP was very close to the sum of the decreases in NADPH, NADH, and NAD.

Iron dependence of the changes in pyridine nucleotides. The killing of cultured hepatocytes by TBHP depends on a cellular source of ferric iron [11, 20]. Thus, the ferric iron chelator deferoxamine prevents the cell killing by TBHP [11, 20]. Figures 3 and 4 illustrate the effect of iron chelation on the metabolism of pyridine nucleotides in hepatocytes intoxicated with TBHP.

Hepatocytes in culture for 24 hr were treated 1 mM TBHP with or without a 1-hr pretreatment with 12 mM deferoxamine. Within 60 min in the absence of the deferoxamine pretreatment, 72% of the cells were dead, whereas only 3% of the cells were dead with deferoxamine. Figure 3 illustrates

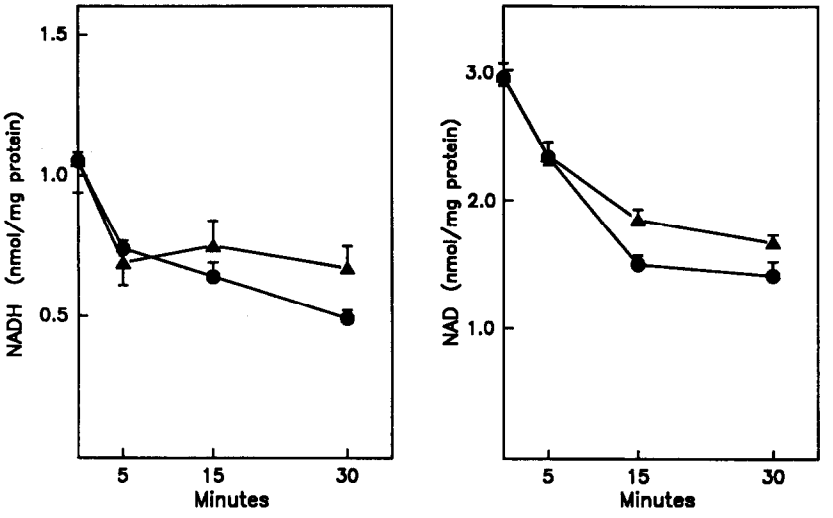

Fig. 4. Effect of pretreatment with the iron chelator deferoxamine on changes in the content of NADH and NAD induced by *tert*-butyl hydroperoxide. Hepatocytes in culture for 24 hr were washed, placed in fresh complete Williams E medium with (▲) or without (●) 12 mM deferoxamine. After 1 hr the cultures were washed, placed in fresh incomplete Williams E medium (no bovine serum), and treated with 1 mM TBHP. At the times indicated, the cellular contents of NADH (left panel) and NAD (right panel) were determined. Results are the means ± SD of determinations on three separate cultures. The difference between the NADH and NAD content at 30 min with and without deferoxamine pretreatment was significant at $P < 0.025$.

the changes in the NADPH (left panel) and NADP (right panel) during the first 30 min of exposure of the hepatocytes to TBHP with or without the pretreatment with deferoxamine. In both cases, the content of NADPH decreased and the content of NADP increased during the first 5 min. However, in the presence of deferoxamine, there was a reaccumulation of NADPH within 30 min. In addition, with the deferoxamine pretreatment, the content of NADP was significantly less than with TBHP alone after 30 min. Figure 4 illustrates that there was a small but significant sparing of the depletion of both NADH (left panel) and NAD (right panel) in the hepatocytes pretreated with deferoxamine. The extent of the sparing of the depletion of NADP (0.27 nmol/mg protein) was the same as that of NAD (0.26 nmol/mg protein).

Mechanism of the increase in NADP. Glutathione peroxidase reduces TBHP to *tert*-butanol, a reaction accompanied by the oxidation of glutathione. In turn, GSSG is reduced by the action of glutathione reductase. The cofactor for this latter reaction is NADPH, which is oxidized to NADP. Thus, the activation of glutathione reductase upon the metabolism of TBHP by glutathione peroxidase should account, at least in part, for the increase in the content of NADP.

BCNU is an inhibitor of the glutathione reductase activity of cultured hepatocytes [21]. Table 2 details the effect of BCNU on the changes in NADPH and NADP in hepatocytes treated with 0.5 mM TBHP, a lower concentration than used in the previous experiments since BCNU potentiates the toxicity of TBHP. In the hepatocytes pretreated with 300 μM BCNU, the magnitude of the changes in both NADPH and NADP was decreased, in each case by 50% (Table 2). Thus, in the presence of BCNU, changes in NADPH and NADP were not as great as with TBHP alone, despite the fact that a greater number of cells eventually died in the presence of BCNU.

Table 2. Effect of BCNU on the changes in NADPH and NADP produced by TBHP

	NADPH	NADP
	(nmol/mg protein)	
	Minus BCNU	
Initial value	1.49 ± 0.04	0.24 ± 0.01
5 min	0.91 ± 0.05	1.43 ± 0.06
Change	−0.58	+1.19
	Plus BCNU	
Initial value	1.46 ± 0.02	0.28 ± 0.01
5 min	1.15 ± 0.04	0.81 ± 0.01
Change	−0.31	+0.53

Cultured hepatocytes pretreated with BCNU were exposed to a 300 μM concentration for 15 min. All cultures were treated with 0.5 mM TBHP for 5 min. Results are the means ± SD of determinations on three separate cultures.

NAD can also be converted to NADP by the action of nicotinamide adenine dinucleotide kinase. In the presence of NAD and ATP, this enzyme forms NADP and ADP. The rat liver enzyme has been reported to have a rather high K_m for ATP, a fact that makes the enzyme sensitive to substrate

Table 3. Effect of oligomycin on the TBHP-induced changes in pyridine nucleotides

Treatment	NADPH	NADP (nmol/mg protein)	NAD
Control	1.40 ± 0.04	0.48 ± 0.06	3.14 ± 0.36
TBHP alone	1.06 ± 0.04	1.90 ± 0.07	1.49 ± 0.14
Oligomycin alone	0.79 ± 0.01	0.36 ± 0.02	2.08 ± 0.27
TBHP + oligomycin	0.70 ± 0.05	1.00 ± 0.02	1.62 ± 0.10

Cultured hepatocytes were pretreated with 0.1 μg/mL of oligomycin for 60 min. Treatment with 1.0 mM TBHP was in the presence of 1 μM DPPD for 30 min. Results are the means ± SD of determinations on three separate cultures.

level inhibition as a consequence of a depletion of ATP. Cultured hepatocytes can be readily depleted of ATP by treatment with oligomycin, and Table 3 shows the effect of such a treatment on the changes in pyridine nucleotides induced by TBHP. In the presence of oligomycin, the increase in NADP and the decrease in NAD were less than in the presence of TBHP alone.

Effect of 3-aminobenzamide on NAD metabolism. Figure 5 shows the concentration–response relationship between TBHP and the cellular content of NAD in the presence or absence of 10 mM 3-aminobenzamide, a concentration that maximally inhibited poly(ADP-ribose) polymerase in cultured hepatocytes.* 3-AB has been reported to protect against oxidative cell injury in other cell systems [8, 9].

TBHP maximally depleted NAD at the lowest concentration examined. Increasing concentrations had little or no further effect on the content of NAD. 3-AB did not prevent the depletion of NAD by TBHP. Since the control level was somewhat higher in the presence of 3-AB, the content of NAD with each concentration of TBHP was higher than in the absence of 3-AB. There was no effect of the 10 mM 3-AB on the killing of the hepatocytes by any of the concentrations of TBHP used in Fig. 5 (data not shown).

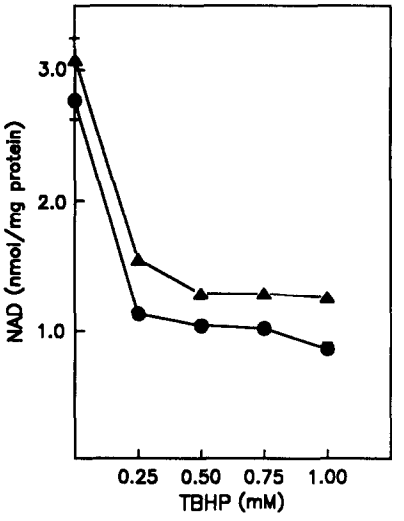

Fig. 5. Effect of 3-aminobenzamide on the loss of NAD induced by *tert*-butyl hydroperoxide. Hepatocytes in culture for 24 hr were washed, placed in fresh incomplete Williams E medium (no bovine serum), and treated with the indicated concentrations of TBHP in the presence (▲) or absence (●) of 10 mM 3-aminobenzamide. After 30 min, the cellular content of NAD was determined. Results are the means ± SD of determinations on three separate cultures. The NAD content in the absence of 3-AB was significantly ($P < 0.001$) lower than in the presence of 3-AB with all concentrations of TBHP.

DISCUSSION

Several conclusions readily emerge from the data presented above. The alterations in NADPH, NADP, NADH, and NAD that accompany the exposure of cultured hepatocytes to TBHP can be dissociated from the appearance of lethal cell injury. In the presence of the antioxidant DPPD, the same pattern of change in the content of pyridine nucleotides developed. However, the cells did not die over the time course that the cells died in the absence of DPPD (Figs. 1 and 2). Alterations in pyridine nucleotide metabolism may not even be necessary for cell killing, a conclusion suggested by the fact that BCNU reduced the pyridine nucleotide changes (Table 2) at the same that it increased the cell killing. Regardless of the necessity of these changes, they are clearly not sufficient to produce lethal cell injury. In other words, changes in the content of NADPH, NADP, NADH, and NAD do not lead to an inexorable sequence of consequences that result in irreversible cell injury.

The changes in response to TBHP do indicate a rapid interconversion of the respective pyridine nucleotides, with little or no change in the total content of these species (Table 1). The initial response seems to reflect, at least in large part, the activation of glutathione reductase with the consequent oxidation of NADPH to NADP. There is a prompt accumulation of GSSG upon exposure to TBHP [11], and inhibition of this enzyme with BCNU decreases in parallel the loss of NADPH and the increase in NADP (Table 3). Finally, similar to the changes in glutathione metabolism [11], the

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initial alterations in the content of pyridine nucleotides are not iron dependent (Figs. 3 and 4).

The increase in NADP exceeded the loss of NADPH (Fig. 1 and Table 1). Interestingly, the increase in NADP equalled the sum of the losses of the other species that were quantified, namely NADPH, NADH, and NAD (Table 1). This would strongly suggest interconversion among the various pyridine nucleotides, a response to oxidative stress previously reported [22]. The effect of oligomycin on the changes in pyridine nucleotides induced by TBHP (Table 3) supports such a conclusion.

In the rat liver, NAD can be converted to NADP by the action of nicotinamide adenine dinucletotide kinase [23]. The K_m for ATP is 7.0 mM, a value suggesting that the enzyme is susceptible to substrate level inhibition when the concentration of ATP is reduced. Depletion of ATP by treatment of the hepatocytes with oligomycin reduced by 3.5-fold the depletion of NAD and 2-fold the increase in NADP. Thus, conversion of NADH to NAD and then NAD to NADP, by nicotinamide adenine dinucleotide kinase, probably accounts for the increase in NADP over that resulting from the oxidation of NADPH by glutathione reducatase. A similar activation of NAD kinase upon exposure of suspensions of isolated rat hepatocytes to three quinones, menadione, 2-3-dimethoxy-1,4-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone, was previously postulated to account for the rapid fall in NAD and the coincident rise in NADP [22]. Interestingly, rat liver NAD kinase is inhibited by NADPH [24]. Thus, NADPH depletion itself can result in NAD kinase activation, owing to the removal of an inhibitory effect of this nucleotide on the enzyme. It deserves emphasis that all these changes in the metabolism of the pyridine nucleotides occur in the absence of any loss of viability of the cultured hepatocytes.

The limited iron dependence (Figs. 3 and 4) of the alterations in pyridine nucleotides induced by TBHP deserves comment. Whereas the antioxidant DPPD was without effect on the changes in pyridine nucleotides (Figs. 1 and 2), the pretreatment with the ferric iron chalator deferoxamine did have an effect (Figs. 3 and 4). Ferric iron chelation prevents the formation of a potent oxidizing species, presumably the hydroxyl radical or, in the case of TBHP, the *tert*-butyl alkoxyl radical. In turn, these radicals are a very reactive species and can have a number of biochemical effects. In this regard, whereas DPPD readily prevents the peroxidation of cellular lipids initiated by the hydroxyl or *tert*-butyl alkoxyl radical, it is without effect on the iron-dependent appearance of single-strand breaks in DNA. In other words, in the presence of DPPD, the *tert*-butyl alkoxyl radical can still interact with cellular targets in ways distinct from the initiation of the peroxidation of membrane phospholipids. It is not unreasonable that these oxidative events were accompanied by the changes in pyridine nucleotides that were prevented by iron chelation (Figs. 3 and 4). Importantly, these changes occurred in the presence of DPPD. Thus, they are either unrelated to the genesis of lethal cell injury, or they may be related to the events that result in the initiation and propagation of the peroxidative decomposition of membrane lipids.

Finally, there is no evidence in cultured hepatocytes intoxicated with TBHP for significant changes in pyridine nucleotides that can be related to an activation of poly(ADP-ribose) polymerase. Two points argue for this conclusion. First, there was little or no net loss of pyridine nucleotides over the time course that there were substantial changes in the contents of the individual species (Table 1). Second concentrations of 3-AB that maximally inhibited poly(ADP-ribose) polymerase in the cultured hepatocytes did not spare the depletion of NAD (Fig. 5) and did not prevent the cell killing by TBHP.

3-AB was reported to prevent the depletion of NAD, but not ATP, and delay the killing of suspended hepatocytes exposed to the hydrogen peroxide generated with glucose oxidase [10]. The reason for this difference between the effects of 3-AB here (Fig. 5) and those reported previously [10] may relate to a difference between TBHP and H_2O_2. 3-AB can scavenge hydroxyl, but not *tert*-butyl alkoxyl, radicals.* Thus, 3-AB may protect from H_2O_2 by a mechanism that is unavailable to cells exposed to TBHP.

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