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DIFFERING EFFECTS OF THE INHIBITION OF POLY(ADP-RIBOSE) POLYMERASE ON THE COURSE OF OXIDATIVE CELL INJURY IN HEPATOCYTES AND FIBROBLASTS

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Abstract—The effects of the two inhibitors of poly(ADP-ribose) polymerase, 3-aminobenzamide (ABA) and benzamide (BA), on the oxidative killing of L929 mouse fibroblasts and primary cultures of rat hepatocytes were studied. The killing of L929 cells by *tert*-butyl hydroperoxide (TBHP) occurred by two mechanisms, one sensitive and the other insensitive to the antioxidant *N,N'*-diphenylphenylene diamine (DPPD). Cell killing by either mechanism was prevented by the ferric iron chelator deferoxamine. ABA and BA prevented the killing of L929 cells that occurred in the presence, but not in the absence, of DPPD. ABA and BA inhibited the activity of poly(ADP-ribose) polymerase by 85%. Protection was accompanied by the sparing of the depletion of both NAD and ATP, but there was no effect of either ABA or BA on the iron-dependent appearance of single-strand breaks in DNA. Depletion of ATP by treating the fibroblasts with 2-deoxyglucose and sodium azide did not result in any loss of viability. H_2O_2 similarly killed the L929 cells by a mechanism that depended on a source of ferric iron. However, DPPD had no effect on the cell killing, and ABA and BA completely protected the cells in the presence or absence of DPPD. H_2O_2 caused the appearance of single-strand breaks that were prevented by deferoxamine, but again not by ABA or BA. ABA and deferoxamine reduced, but did not prevent, the depletion of both NAD and ATP occurring with H_2O_2. With the cultured hepatocytes, ABA and BA inhibited poly(ADP-ribose) polymerase at concentrations that were without effect on either the extent of cell killing or the depletion of NAD occurring with either TBHP, H_2O_2, or menadione. These data indicate that the relationship between oxidative DNA damage and the genesis of lethal injury is very different in the two types of cells. In the fibroblasts, the appearance of single strand breaks in DNA was accompanied by depletion of NAD and ATP and subsequently by the death of the cells. These events were mediated by the activity of poly(ADP-ribose) polymerase, as inhibition of the enzyme prevented their development. In the hepatocytes, inhibition of poly(ADP-ribose) polymerase was without effect on the oxidative death of the cells.

The appearance of single-strand breaks in DNA is a common manifestation of the exposure of cells to an oxidative stress [1–5]. In turn, the repair of these lesions is accompanied by activation of poly(ADP-ribose) polymerase, a nuclear enzyme that catalyzes the transfer of ADP-ribose from NAD to protein with the concomitant release of nicotinamide.

Poly(ADP-ribose) polymerase has been implicated in the lethal cell injury produced by an oxidative stress. In particular, it has been proposed that the metabolic consequences of the activation of this enzyme, namely the consumption of NAD and a consequent depletion of ATP, are involved in the pathogenesis of oxidant-induced cell death [4, 6, 7].

The evidence supporting such an hypothesis derived from studies of the killing of human lymphocytes [4], P388D1 cells [4, 6], a murine macrophage tumor cell line, and endothelial cells [7] by activated oxygen species. 3-Aminobenzamide (ABA§), an inhibitor of poly(ADP-ribose) polymerase, prevented the loss of viability caused by the oxidative stress [4, 7]. At the same time, ABA prevented the depletion of NAD and ATP [4, 7]. Thus, it was suggested that activation of poly(ADP-ribose) polymerase following oxidant-induced DNA single-strand breaks results in NAD depletion and a consequent loss of ATP [4, 7]. Such a role for poly(ADP-ribose) polymerase activation has also been implicated in the killing of isolated hepatocytes by H_2O_2 [8], as well as in the toxicity of the diabetogenic agents alloxan and streptozotocin [9, 10].

We have previously detailed the changes in the metabolism of pyridine nucleotides in cultured hepatocytes intoxicated with *tert*-butyl hydroperoxide (TBHP) [11], a useful model with which to study the mechanisms of oxidative cell injury [12–14]. The loss of NADPH, NADH, and NAD equalled the increase in NADP, with little if any decrease in the total content of pyridine nucleotides. The changes were interpreted as indicating a rapid interconversion of the respective species, the initial response reflecting activation of glutathione reductase and the

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§ Abbreviations: ABA, 3-aminobenzamide; BA, benzamide; DMSO, dimethyl sulfoxide; DPPD, *N,N'*-diphenylphenylene diamine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; and TBHP, *tert*-butyl hydroperoxide.

consequent oxidation of NADPH to NADP. The conversion of NADH to NAD and then NAD to NADP, the latter by nicotinamide adenine dinucleotide kinase, could account for the increase in NADP over that resulting from the oxidation of NADPH by glutathione reductase. There was no evidence in the cultured hepatocytes treated with TBHP that the loss of NAD reflected the activation of poly(ADP-ribose) polymerase, and ABA did not protect the cells.

The present report attempts to resolve this apparent discrepancy between the role of poly(ADP-ribose) polymerase in oxidative cell injury in cultured hepatocytes [11] as opposed to other cell types [4, 6–8]. In particular, we have compared the effects of inhibitors of poly(ADP-ribose) polymerase, ABA and benzamide (BA), on the killing of L929 mouse fibroblasts and rat heptocytes by both TBHP and H_2O_2. The data document that single-strand breaks in DNA are associated with very different consequences in the two cell types. In the fibroblasts, inhibition of poly(ADP-ribose) polymerase protected against the loss of viability occurring with both TBHP and H_2O_2. By contrast, inhibition of this enzyme in hepatocytes had little effect on the cell killing by TBHP and H_2O_2.

MATERIALS AND METHODS

The murine fibroblast L929 cell line was purchased from the American Type Tissue Collection (Rockville, MD) and maintained in 25 cm^2 flasks (Corning Glass Works, Corning, NY) with 5 mL of minimum essential medium containing non-essential amino acids (GIBCO Laboratories, Grand Island, NY), 50 IU/mL penicillin, 50 μg/mL streptomycin and 10% heat-inactivated (55° for 15 min) fetal bovine serum (JRH Biosciences, Kansas City, MO) under an atmosphere of 95% air–5% CO_2. The cells were subcultured once a week with the use of 0.25% trypsin. Experiments were performed 2 days after plating, at a time when the cells had achieved a density of approximately 2.0×10^6 cells/flask. The cells were washed and incubated in 5 mL of fresh medium for 30 min and then treated as indicated in the text. For the cytotoxicity assays, the cells were plated in 24 culture well plates (Becton Dickinson & Co.) with 0.5 mL of medium at a density of 2.0×10^5 cells/well. Viability was defined as the ratio of the lactate dehydrogenase (LDH) in the medium to the total that could be released from the culture by Triton X-100 under the same conditions. This corrected for any effect of the treatment on the growth of the cells or on the content of LDH. All experiments were repeated at least three times on triplicate cultures.

Male Sprague–Dawley rats (150–200 g) were obtained from the Charles River Breeding Laboratory (Wilmington, MA). The animals were fed *ad lib.* and fasted overnight prior to use. Isolated hepatocytes wre prepared by collagenase (Boehringer Mannheim Biochemicals) perfusion according to Seglen [15]. Yields of 2–4 $\times 10^8$ cells per liver with 90–95% viability (as determined by trypan blue exclusion) were routinely obtained. The hepatocytes were plated on 25 cm^2 plastic flasks (Corning) at a density of 1.33×10^6 cells per flask in 3 mL of Williams E medium (GIBCO) containing 10 IU/mL penicillin, 10 μg/mL streptomycin, 50 μg/mL gentamycin, 0.02 U/mL insulin and 10% heat-inactivated fetal bovine serum (complete Williams E medium). After an incubation of 2 hr at 37° in an atmosphere of 95% air–5% CO_2, the cultures were rinsed twice with 3 mL of prewarmed 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (142 mM NaCl, 6.7 mM KCl, 1.3 mM $CaCl_2$ and 10.1 mM HEPES, pH 7.4) to remove any unattached or dead cells. Fresh complete Williams E medium (5 mL) was replaced, and the cells were incubated for 18 hr. The cultures were then washed and incubated in Williams E minus fetal bovine serum (incomplete Williams E) with the chemicals indicated in the text.

TBHP (Sigma) and H_2O_2 (Sigma) were dissolved in the culture medium and added to the cells at the concentrations indicated in the text. Menadione (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and added at a concentration of 200 μM (final DMSO concentration was 0.3%). Deferoxamine (CIBA Pharmaceutical Co.) was dissolved in distilled water. The cells were pretreated for 1 hr with 12 mM deferoxamine. The medium was then changed, and 1 mM deferoxamine was added for the duration of the exposure to either TBHP or H_2O_2. *N,N'*-Diphenylphenylene diamine (DPPD) (Aldrich Chemical Co.) was dissolved im DMSO, diluted with medium, and added to the cultures at a final concentration of 1 μM (final DMSO concentration was 0.005%). BA (Sigma) and ABA (Sigma) were dissolved in medium and added to the cultures at the concentrations indicated in the text.

The content of NAD and other pyridine nucleotides was determined by the methods described previously [11]. ATP content was determined with the use of luciferin-luciferase as described previously [16]. Lipid peroxidation, as determined by the accumulation of malondialdehyde (MDA) in the cultures, was measured by a modification of the method of Ohkawa *et al.* [17]. DNA single-strand breaks were measured by the fluorometric analysis of the rate of DNA unwinding according to the previously described modifications [14] of the method of Birnboim and Jevcak [18]. The activity of poly(ADP-ribose) polymerase was determined by the method of Althaus *et al.* [19]. All experiments were repeated at least three times with triplicate cultures.

RESULTS

Killing of L929 cells by TBHP and H_2O_2. As shown in Fig. 1, the killing of L929 fibroblasts by TBHP was dependent on a source of ferric iron. The "left panel" details the effect of the ferric iron chelator deferoxamine on the killing of L929 fibroblasts by TBHP. In the absence of deferoxamine, over 80% of the cells were killed by 1 mM TBHP within 6 hr. Over the same time course, deferoxamine reduced the killing to less than 10% of the cells.

Figure 1 also illustrates the effect of deferoxamine on the time course of the changes produced by TBHP in the contents of NAD (middle panel) and ATP (right panel). Whereas cell viability was

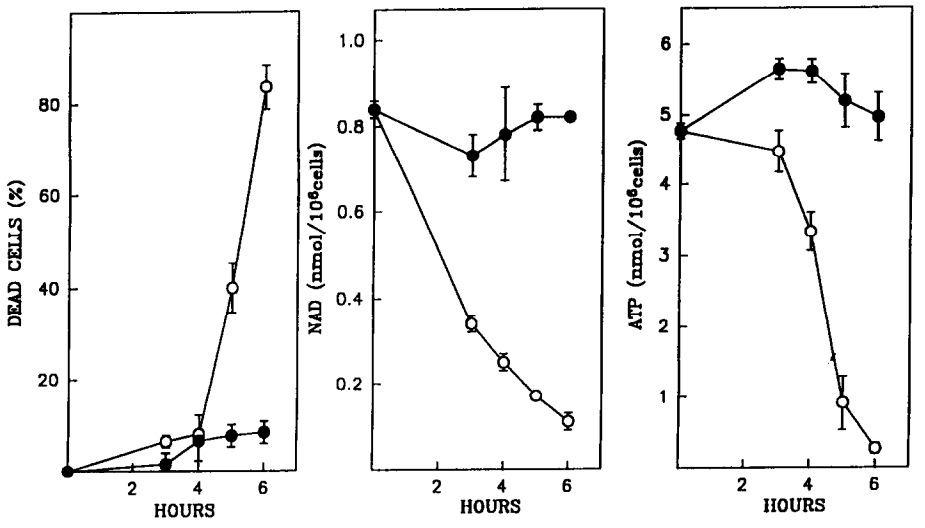

Fig. 1. Effect of deferoxamine on the time course of the changes in viability and the contents of NAD and ATP in L929 fibroblasts treated with TBHP. Cells exposed to deferoxamine (●) were pretreated with 12 mM deferoxamine for 1 hr and then resuspended in fresh medium containing 1 mM deferoxamine and 1 mM TBHP. Cells not exposed to deferoxamine (○) were not pretreated. The medium was changed, and the cells were resuspended in fresh medium containing 1 mM TBHP. Results are the means ± SD of the determination on three separate cultures.

maintained for over 4 hr, the content of NAD was reduced by 60% within 3 hr. At the end of 6 hr, the NAD content declined to less than 15% of the initial value. Deferoxamine maintained the content of NAD over the entire 6-hr time course of the experiment. ATP depletion, evident within 4 hr, followed that of NAD, but again preceded the cell killing. Deferoxamine maintained the content of ATP throughout the 6 hr of the experiment.

The killing of L929 fibroblasts by TBHP occurred by at least two mechanisms, one sensitive and the other insensitive to an antioxidant. Figure 2 illustrates the cell killing that occurred after 6 hr with increasing concentrations of TBHP in the presence or absence of the antioxidant DPPD. Without DPPD cell killing was maximal with 1 mM TBHP. Whereas 1 mM TBHP killed 85% of the cells in the absence of DPPD, in its presence the same dose of TBHP killed less than 50% of the cells. With DPPD in the medium, cell killing was less with every dose of TBHP.

The killing of the L929 cells in the absence of DPPD was accompanied by the peroxidation of membrane lipids, as evidenced by an accumulation of MDA in the cultures (data not shown). Protection of the cells by deferoxamine and DPPD was accompanied by an inhibition of this lipid peroxidation (data not shown).

The effect of 1 mM ABA on the killing of L929 cells by TBHP in the absence or the presence of DPPD is illustrated in Figs. 3 and 4, respectively. In the absence of DPPD (Fig. 3, left panel), ABA delayed, but did not substantially prevent, the toxicity of 1 mM TBHP. Within 6 hr cell killing in the presence of ABA was 80% of its extent in the absence of ABA. ABA also delayed the depletion

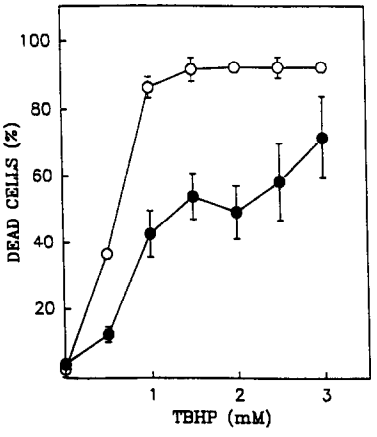

Fig. 2. Effect of DPPD on the killing of L929 fibroblasts by TBHP. The cells were exposed to increasing concentrations of TBHP in the presence (●) or absence (○) of 1 μM DPPD. The viability of the cells was determined after 6 hr. Results are the means ± SD of the determinations on three separate cultures.

of NAD (Fig. 3, middle panel), but had very little effect on the loss of ATP (Fig. 3, right panel).

By contrast, in the presence of DPPD, ABA completely prevented the cell killing by 1.5 mM TBHP (Fig. 4, left panel). Whereas after 7 hr almost 90% of the cells were dead in the absence of ABA, cell killing in its presence was no greater than in control cultures. Under these same conditions, ABA

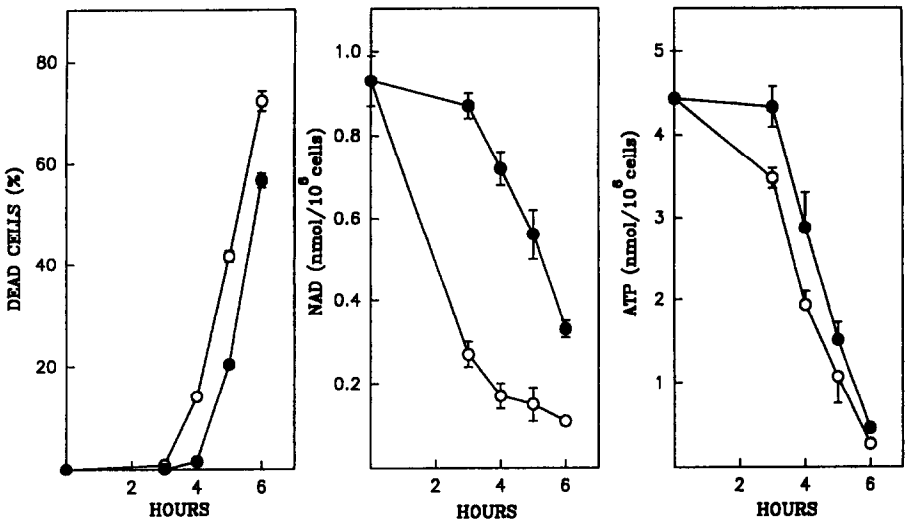

Fig. 3. Effect of 3-aminobenzamide on the time course of the changes in viability and the contents of NAD and ATP in L929 fibroblasts treated with TBHP. The cells were treated with 1 mM TBHP in the presence (●) or absence (○) of 1 mM ABA. Results are the means ± SD of the determinations on three separate cultures.

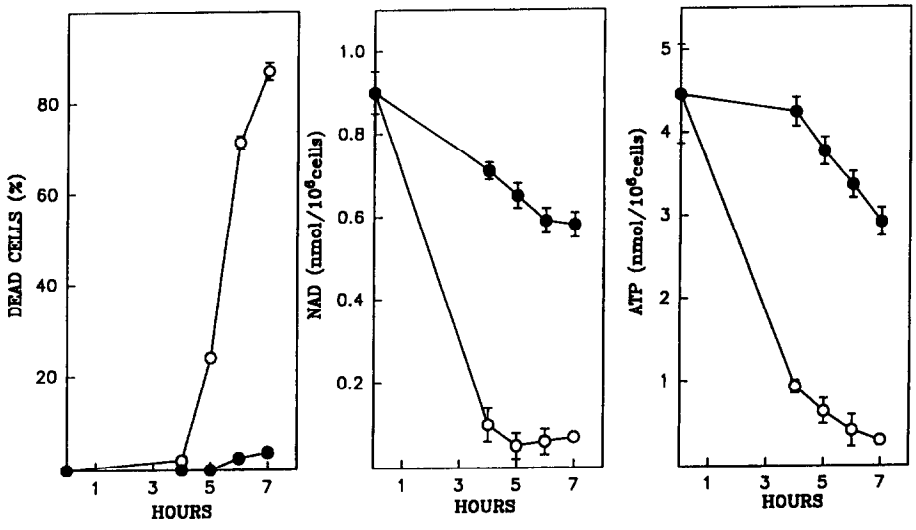

Fig. 4. Effect of 3-aminobenzamide on the time course of the changes in viability and the contents of NAD and ATP in L929 fibroblasts treated with TBHP in the presence of DPPD. The cells were treated with 1.5 mM TBHP and 1 μM DPPD in the presence (●) or absence (○) of 1 mM ABA. Results are the means ± SD of the determinations on three separate cultures.

reduced the extent of the depletion of NAD (Fig. 4, middle panel) and ATP (Fig. 4, right panel).

The killing of the fibroblasts in the absence of lipid peroxidation (i.e. in the presence of DPPD) cannot be attributed to the depletion of ATP alone (Fig. 4, right panel). Figure 5 shows the time course of the depletion of ATP achieved by treating the cells with 2-deoxyglucose and sodium azide. Whereas there was a more rapid loss of ATP than occurred with TBHP, there was no loss of viability at the end of 6 hr.

Both ABA and BA inhibit the poly(ADP-ribose) polymerase activity present in L929 fibroblasts. Table 1 documents that 1 mM ABA and BA inhibited poly(ADP-ribose) polymerase by 85%. Table 2 shows that benzamide was also a potent inhibitor of the killing of L929 cells by TBHP. As with ABA, BA had no effect on cell killing in the absence of DPPD (line 2). However, in the presence of DPPD, BA reduced the cell killing to less than 10% of the cells (line 4). Importantly, neither ABA nor BA prevented the appearance of single-strand breaks

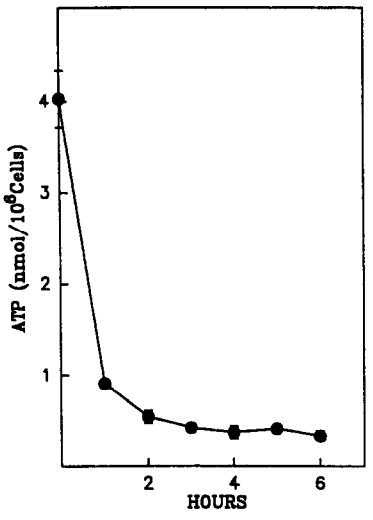

Fig. 5. Time course of the depletion of ATP in L929 fibroblasts treated with 100 mM 2-deoxyglucose and 1 mM sodium azide. Results are the means ± SD of the determinations of three separate cultures.

Table 1. Inhibition by 3-aminobenzamide and benzamide of the poly(ADP-ribose) polymerase activity of L929 cells

Treatment	Poly(ADP-ribose) polymerase activity (% of control)
3-Aminobenzamide (1 mM)	16.1 ± 2.6
Benzamide (1 mM)	15.7 ± 3.6

The absolute value of the activity of the enzyme was 0.1 nmol adenine incorporated/10^6 cells. Results are the means ± SD of three separate determinations.

Table 2. Prevention by benzamide of the killing of L929 cells by *tert*-butyl hydroperoxide

Treatment	Dead cells (%)
1. TBHP (1 mM)	74.4 ± 4.3*
2. TBHP (1 mM) + BA (1 mM)	77.3 ± 5.5*
3. TBHP (1.5 mM) + DPPD (1 μM)	56.9 ± 3.1†
4. TBHP (1.5 mM) + DPPD + BA	8.1 ± 3.3†

Results are the means ± SD of the determinations on three separate cultures.

* Viability was determined 6 hr after addition of TBHP.

† Viability was determined 7 hr after addition of TBHP.

in DNA (Table 3). By contrast, deferoxamine completely prevented the appearance of such lesions in DNA (Table 3).

ABA and BA also prevent the iron-dependent killing of L929 fibroblasts by hydrogen peroxide

Table 3. Effects of deferoxamine, 3-aminobenzamide, and benzamide on DNA single-strand breaks and cell death produced in L929 cells by *tert*-butyl hydroperoxide

Treatment	Double-stranded DNA (%)	Dead cells (%)
Control	88.5 ± 1.5	0.0 ± 1.0
TBHP (1 mM)	55.3 + 8.9	90.4 ± 4.4
TBHP + DEF	79.7 ± 4.0	2.0 ± 0.0
TBHP + ABA (1 mM)	52.8 ± 8.6	80.2 ± 3.2
TBHP + BA (1 mM)	52.9 ± 3.9	99.4 ± 2.0
TBHP + DPPD (1 μM)	53.1 ± 0.8	38.3 ± 3.9
TBHP + DPPD + ABA	49.1 ± 7.6	6.4 ± 1.3
TBHP + DPPD + BA	39.2 ± 6.2	1.8 ± 4.1

L929 cells were incubated with TBHP and the additions indicated. DNA single-strand breaks and cell death were examined at 3 and 6 hr, respectively. Results are the means ± SD of the determinations on three separate cultures.

Table 4. Prevention by 3-aminobenzamide and benzamide of the killing of L929 cells by H_2O_2

Treatment	Dead cells (%)
1. H_2O_2 (2 mM)	89.9 ± 8.5
2. H_2O_2 + DEF	4.6 ± 9.8
3. H_2O_2 + DPPD (1 μM)	69.8 ± 2.4
4. H_2O_2 + ABA (1 mM)	1.0 ± 7.0
5. H_2O_2 + BA (1 mM)	2.0 ± 1.7
6. H_2O_2 + DPPD + ABA	1.0 ± 8.7
7. H_2O_2 + DPPD + BA	7.0 ± 3.5

Results are the means ± SD of the determinations on three separate cultures.

(Table 4). In contrast to TBHP, cell killing by H_2O_2 occurred largely in the absence of lipid peroxidation, as indicated by the fact that DPPD had a limited protective effect (comparison of Table 4, lines 1 and 3). Thus, ABA (lines 4 and 6) and BA (lines 5 and 7) protected from the cell killing in the absence or presence of DPPD.

Figure 6 (left panel) illustrates the effect of deferoxamine and ABA on the time course of the killing of L929 cells by H_2O_2. Both deferoxamine and ABA reduced the cell killing after 6 hr from more than 80% of the cells in their absence to less than 10% of the cells in their presence.

Figure 6 also shows the effect of both deferoxamine and ABA on the time course of the depletion of NAD (middle panel) and ATP (right panel) produced by 2 mM H_2O_2. Several features of the data are noteworthy. Whereas the cell killing by H_2O_2 was entirely iron dependent, the decreases in both NAD and ATP were not. ABA and deferoxamine spared, but did not prevent, the depletion of NAD and ATP. However, the effects of deferoxamine and ABA on the content of NAD and ATP were identical.

These results contrast with the effect of TBHP,

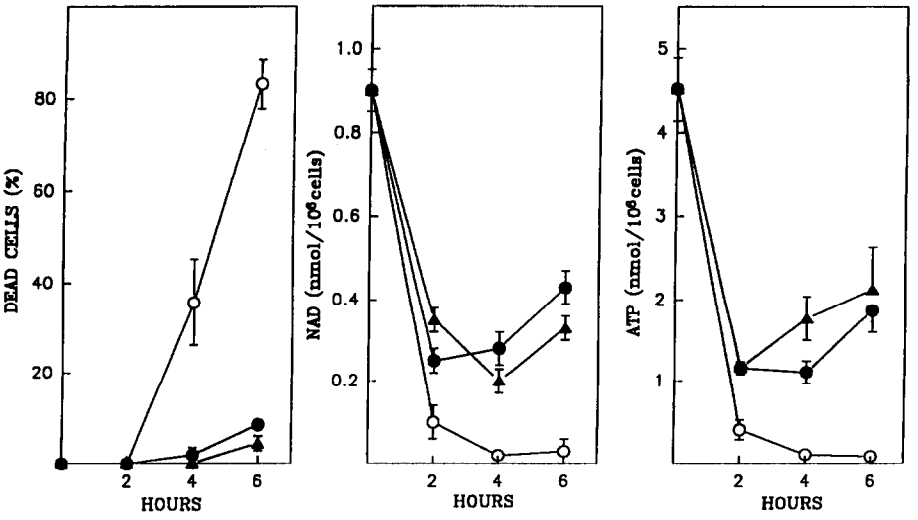

Fig. 6. Effects of deferoxamine and 3-aminobenzamide on the time course of the changes in viability and the contents of NAD and ATP in L929 fibroblasts treated with H_2O_2. Cells exposed to deferoxamine (●) were pretreated with 12 mM deferoxamine for 1 hr and then resuspended in fresh medium containing 1 mM deferoxamine and 2 mM H_2O_2. Other cells were exposed to 2 mM H_2O_2 alone (○) or to 2 mM H_2O_2 in the presence of 1 mM ABA (▲). Results are the means ± SD of the determinations of three separate cultures.

Table 5. Effects of deferoxamine, 3-aminobenzamide, and benzamide on DNA single-strand breaks produced in L929 cells by H_2O_2

Treatment	% Double-stranded DNA
1. Control	72.9 ± 4.5
2. H_2O_2 (2 mM)	35.9 ± 2.4
3. H_2O_2 + DEF	74.8 ± 4.6
4. H_2O_2 + ABA (1 mM)	33.0 ± 8.4
5. H_2O_2 + BA (1 mM)	37.0 ± 8.4

L929 cells were incubated with H_2O_2 and the additions indicated. DNA single-strand breaks were quantitated 1 hr after treatment with H_2O_2. Results are the means ± SD of the determinations of three separate cultures.

where the depletion of NAD and ATP was both entirely iron dependent (Fig. 1) and prevented to a much greater extent by ABA in the presence of DPPD (Fig 4). Furthermore, comparison of Figs. 1 and 6 indicates that the fibroblasts reacted faster to H_2O_2 than to TBHP. Within 2 hr with H_2O_2, the depletion of both NAD and ATP was largely complete, and cell killing occurred within 4 hr.

The appearance of single-strand breaks in DNA occurring with H_2O_2 was entirely iron dependent (Table 5), as indicated in its prevention by deferoxamine (lines 2 and 3). Whereas ABA and BA similarly prevented the cell killing by H_2O_2 (Table 4, lines 4 and 5), neither poly(ADP-ribose) polymerase inhibitor prevented the appearance of single-strand breaks in DNA.

Similar to the change in NAD, the depletion of NADPH and NADH that occurred with H_2O_2 was also largely iron dependent (data not shown). By contrast, with TBHP the depletion of NADH and NADPH was iron dependent (data not shown), similar to the loss of NAD.

Killing of cultured hepatocytes by TBHP. We have shown previously that ABA does not prevent the killing of cultured hepatocytes by TBHP [11]. However, these studies were performed in the absence of DPPD, and a situation similar to that illustrated in Figs. 3 and 4 may result. Figure 7 illustrates the effect of 1 and 10 mM ABA on hepatocytes treated with 1.5 mM TBHP in the presence of DPPD. The presence of ABA at either 1 or 10 mM had no effect on the cell killing (left panel), the loss of NAD (middle panel), and the loss of ATP (right panel). Similar results were obtained with 1 and 10 mM BA (data not shown). The inability of ABA and BA to prevent the cell killing by TBHP in hepatocytes occurred despite the ability of both compounds to inhibit poly(ADP-ribose) polymerase. Figure 8 shows that 1 mM ABA and 1 mM benzamide inhibited the enzyme by almost 70%.

Table 6 documents that neither ABA nor BA prevented the killing of cultured hepatocytes by H_2O_2. Similarly, neither ABA nor BA had any effect on the killing of cultured hepatocytes by menadione (Table 7).

DISCUSSION

The exposure of both fibroblasts (Tables 3 and 5) and hepatocytes [14] to an oxidative stress is accompanied by the appearance of DNA single-strand breaks. However, as the data presented above show, the effect that these lesions in DNA have on

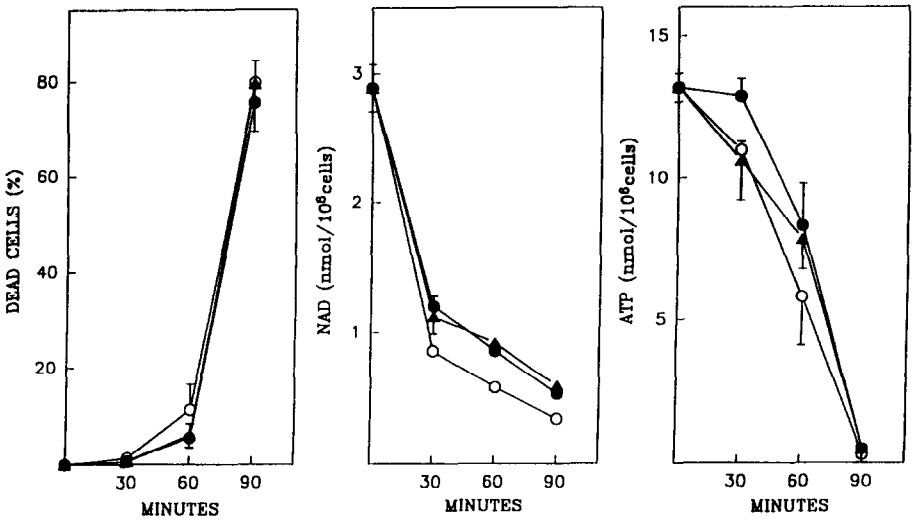

Fig. 7. Effect of 3-aminobenzamide on the time course of the changes in viability and the contents of NAD and ATP in cultured hepatocytes treated with TBHP. The hepatocytes were treated with 1.5 mM TBHP alone (○) or with 1.5 mM TBHP in the presence of 1 mM ABA (●) or 10 mM ABA (▲). Results are the means ± SD of the determinations on three separate cultures.

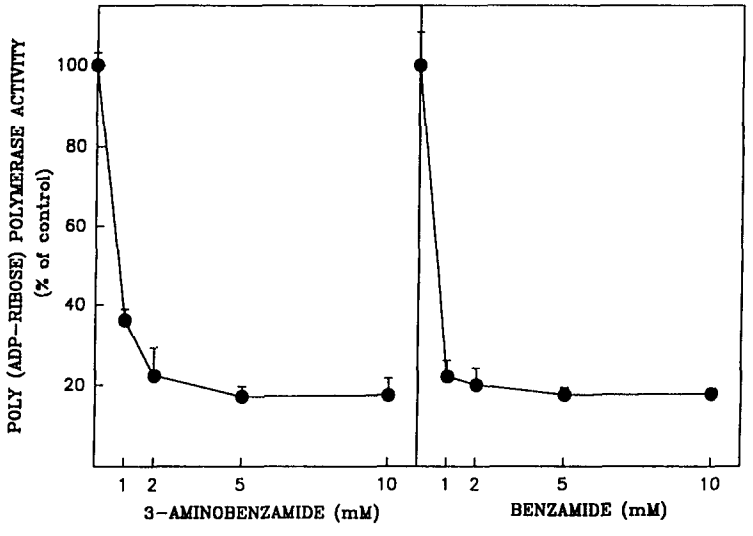

Fig. 8. Inhibition of the poly(ADP-ribose) polymerase activity of cultured hepatocytes by 3-aminobenzamide and benzamide. Cultured hepatocytes were assayed for the enzyme as described in Materials and Methods. The absolute value of the enzyme was 0.3 nmol adenine incorporated/10^6 cells. Results are the means ± SD of the determinations on three separate cultures.

the maintenance of cell viability is very different in the two cell types.

Oxidative cell injury in both fibroblasts (Fig. 1 and Tables 3 and 4) and hepatocytes [12, 20] depends on a cellular source of ferric iron. We have presented data that the ferric iron is reduced to ferrous iron by superoxide anions [20, 21]. In turn, the ferrous iron reacts with H_2O_2 (or TBHP) to produce a more potent oxidizing species (the hydroxyl or *tert*-butyl alkoxyl radical).

Three cellular targets of these radicals have been identified in the hepatocytes. An attack on the phospholipids of cellular membranes initiates their peroxidative decomposition [12, 22]. An attack on the mitochondria results in the loss of the mitochondrial membrane potential and energy depletion [13, 23]. Finally, an attack on DNA results in the appearance of single-strand breaks. We have presented data that the lethal injury of cultured hepatocytes occurring with an oxidative stress is a

Table 6. Cell death and NAD content in hepatocytes treated with H_2O_2

Treatment	Cell death (%)	NAD (nmol/10^6 cells)
Control	1.8 ± 0.0	3.06 ± 0.15
ABA (1 mM)	1.8 ± 0.9	3.63 ± 0.20
ABA (10 mM)	1.2 ± 0.5	3.83 ± 0.16
H_2O_2 + (5 mM)	75.7 ± 1.4	1.38 ± 0.08
H_2O_2 + DEF	15.6 ± 2.4	1.70 ± 0.05
H_2O_2 + DPPD (1 μM)	4.9 ± 0.5	1.02 ± 0.08
H_2O_2 + ABA (1 mM)	75.7 ± 2.3	1.98 ± 0.18
H_2O_2 + ABA (10 mM)	61.6 ± 3.2	2.31 ± 0.10
H_2O_2 + BA (1 mM)	76.0 ± 1.1	2.15 ± 0.14

NAD content and cell death were examined at 30 min and 3 hr after treatment with H_2O_2, respectively. Results are the means ± SD of the determinations on three separate cultures.

Table 7. Cell death and NAD content in hepatocytes treated with menadione

Treatment	Dead cells (%)	NAD (nmol/10^6 cells)
Control	3.7 ± 0.9	2.49 ± 0.01
ABA (1 mM)	4.9 ± 0.5	3.25 ± 0.07
ABA (10 mM)	3.7 ± 0.9	3.43 ± 0.04
Menadione (300 μM)	55.8 ± 1.0	1.40 ± 0.03
Menadione + DEF	43.5 ± 1.9	1.42 ± 0.01
Menadione + DPPD (1 μM)	45.0 ± 3.3	1.42 ± 0.02
Menadione + ABA (1 mM)	41.7 ± 6.3	1.57 ± 0.02
Menadione + ABA (10 mM)	54.3 ± 5.8	1.69 ± 0.03
Menadione + BA (1 mM)	57.0 ± 5.7	1.58 ± 0.05

NAD content and cell death were examined at 30 min and 3 hr after treatment with menadione, respectively. Results are the means ± SD of the determinations on three separate cultures.

result of both the peroxidation of cellular membranes [12, 22] and the de-energization of the mitochondria [13, 23].

By contrast, our previous study [14] and the results of the present one indicate that neither the oxidative damage to DNA nor the activity of poly(ADP-ribose) polymerase in response to the repair of this damage is related to the killing of cultured hepatocytes by an oxidative stress. In the presence of the antioxidant DPPD, cell killing was prevented despite the fact that there was no change in the extent of the accumulation of single-strand breaks in DNA [14]. Inhibitors of poly(ADP-ribose) polymerase have no effect on the killing of cultured hepatocytes by either TBHP (Ref. 11 and Fig. 7) or H_2O_2 (Tables 6 and 7).

As in the hepatocytes, the oxidative injury of L929 fibroblasts depends on ferric iron as shown by the ability of deferoxamine to prevent the killing by both TBHP (Fig. 1, Table 3) and H_2O_2 (Fig. 6 and Table 4). However, in contrast to the situation in hepatocytes, the iron-dependent damage to DNA is clearly a critical event in the genesis of lethal cell injury in the fibroblasts. When TBHP was used to injure the L929 cells oxidatively, the cell killing that occurred in the absence of lipid peroxidation (presence of DPPD) was prevented completely by both ABA (compare Fig. 4 with Fig. 7) and BA (Table 2).

When H_2O_2 was used to injure the L929 cells, there was little cell killing by lipid peroxidation (Table 4), and ABA and BA completely protected these fibroblasts in the presence or absence of DPPD (Table 4). Thus, in contrast to the hepatocytes where its damage is an epiphenomenon [14], in the fibroblasts DNA would seem to be the target of the lethal attack on the cells by H_2O_2.

In the fibroblasts, the chelation of iron by deferoxamine and the inhibition of poly(ADP-ribose) polymerase by BA and ABA had identical consequences with respect to the loss of viability and the depletion of both NAD and ATP (Figs. 1 and 4 with TBHP and Fig. 6 with H_2O_2). However, the action of these agents differed in one important respect. Deferoxamine prevented the appearance of single-strand breaks in DNA, whereas ABA and BA did not. Rather, inhibiton of poly(ADP-ribose) polymerase prevented the consequences of the action of this enzyme upon the appearance of DNA single-strand breaks.

The differing consequences of oxidative damage to DNA in fibroblasts and hepatocytes can be related to another important feature distinguishing these cells. L929 fibroblasts are proliferating cells, whereas hepatocytes are resting cells, which do not normally divide. Single-strand breaks inhibit DNA replication and thereby cellular proliferation. Thus, it might be expected that a proliferating cell, such as the L929 fibroblast, would rapidly repair any damage to its DNA, an activity that requires poly(ADP-ribose) polymerase. By contrast, hepatocytes would be expected to be less attentive to single-strand breaks, since they are not usually replicating their DNA. This conclusion is consistent with the observation that hepatocytes maintain viability in the presence of an antioxidant, despite evidence of DNA damage equivalent to what in the fibroblasts was associated with lethal cell injury [14].

The precise relationship between the activity of poly(ADP-ribose) polymerase and the death of the fibroblasts is not entirely clear. Inhibition of this enzyme not only preserves the viability of the cells, but also maintains the contents of both NAD and ATP. The depletion of these constituents is readily attributable to an exaggerated ADP-ribosylation of proteins, and the loss of viability has been attributed to loss of energy stores [4, 7].

However, the oxidative death of L929 fibroblasts is not attributable to the depletion of ATP itself. By treating the fibroblasts with 2-deoxyglucose and sodium azide, a comparable loss of ATP was achieved (Fig. 5) without any loss of viability. We suspect that the situation here is analogous to that in the hepatocyte, where the mechanism of depletion of ATP determines the fate of the cell. ATP depletion without mitochondrial de-energization is not accompanied by cell death [13], whereas the loss of the mitochondrial membrane potential is generally

associated with a loss of viability [13, 23]. Depletion of NAD would effectively act as a site I inhibitor and result, as with site I inhibition in hepatocytes, in loss of the mitochondrial membrane potential.

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