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Dissociation of the Accumulation of Single-Strand Breaks in DNA from the Killing of Cultured Hepatocytes by an Oxidative Stress

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SUMMARY

The relationship was explored between the accumulation of single-strand breaks in DNA and the killing of cultured hepatocytes by an oxidative stress generated by either *tert*-butyl hydroperoxide (TBHP), glucose oxidase, or menadione. The accumulation of DNA strand breaks was measured fluorometrically by the rate of the alkaline unwinding of DNA. In each case, DNA strand breaks were detected before the loss of cell viability. DNA damage and cell killing depended on a cellular source of iron. Pretreatment of the hepatocytes with the ferric iron chelator deferoxamine prevented both, and the readdition of iron to the medium restored the DNA damage and the cell killing. In addition, the radical scavenger keto-methiolbutyric acid reduced the extent of DNA damage and prevented the cell killing. By contrast, the antioxidants *N,N'*-diphenyl-*p*-phenylenediamine and butylated hydroxytoluene prevented the cell killing but not the DNA single-strand breaks induced by TBHP. Similarly, acidification of the culture medium also prevented the cell killing, without any effect on the extent of the DNA damage by TBHP, glucose oxidase, and menadione. These data indicate that DNA damage and cell killing produced by an oxidative stress depend upon the iron-catalyzed formation of a potent oxidizing species. However, the accumulation of such damage can be dissociated from the mechanisms that lethally injure the cells.

Partially reduced and thereby activated oxygen species, namely superoxide anions, hydrogen peroxide, and the hydroxyl radical, are likely mediators of cell injury in an increasing number of circumstances. Several biochemical alterations have been described and, in turn, proposed as bases for the irreversible injury that may follow exposure of cells to activated oxygen species. In particular, attention has focused on the role of DNA as a target molecule in oxidative cell injury.

A number of different lesions in DNA can be produced by activated oxygen species. Reaction with deoxyribose results in fragmentation with loss of the base and the appearance of a strand break (1–3). Alternatively, reaction with thymine produces a variety of lesions that can be removed by repair enzymes which similarly produce single-strand breaks (1–3). Thus, the appearance of single-strand breaks in DNA is the common consequence of the interaction of oxygen radicals with DNA. Single-strand breaks have been reported to accumulate in bacteria and in a variety of mammalian cells upon exposure to oxygen radicals generated by a number of different mechanisms (1, 4–8).

In turn, oxidative DNA damage has been implicated in the killing of both bacterial and mammalian cells by oxygen radicals. Resistance to hydrogen peroxide was induced in *Escherichia coli* by a prior exposure to nonlethal concentrations of H_2O_2, a result that was accompanied by evidence of the induction of DNA repair activities (8). The generation of an ultimate DNA-damaging species in *E. coli* exposed to hydrogen peroxide depended on a source of ferric iron, and a variety of metal chelators prevented the killing of the bacteria by H_2O_2 (1). Furthermore, mutant strains with defects in the repair of DNA were particularly sensitive to the toxicity of oxygen radicals (10–12).

Mammalian cell lines exposed to H_2O_2 showed a dose-dependent relationship between the accumulation of DNA single-strand breaks and the extent of cell killing (6). Superoxide dismutase decreased the number of thymine glycols produced by benzo(*a*)pyrene in epithelial cells at the same time that it increased cell survival (7). The EM9 mutant of the CHO cell line was more sensitive to the toxicity of H_2O_2, a situation that correlated with a decreased ability of the cells to repair lesions in DNA (9). Finally, inhibition of poly(ADP-ribose) polymerase, an enzyme that is activated by DNA strand breaks, protected P388D_1 cells from the toxicity of hydrogen peroxide (4). This result was interpreted as indicating that the metabolic responses to DNA damage may initiate a sequence of events that eventuate in cell killing (4).

Two concerns are raised by these studies of the relationship

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ABBREVIATIONS: TBHP, *tert*-butyl hydroperoxide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BHT, butylated hydroxytoluene; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; KTBA, α-keto-γ-methiolbutyric acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

between oxidative DNA damage and cell killing. Firstly, manipulations that alter the formation or reactivity of oxygen radicals, such as the use of iron chelators or hydroxyl radical scavengers, have not distinguished between radical-mediated DNA damage and other lesions in the cell that may relate more directly to the loss of viability. Secondly, most previous studies used replicating cell populations and assessed cell killing by an inability of the damaged cells to proliferate. The precise fate of the injured and, thus, nonproliferating cells is not clear. In particular, one can question whether the arrested cells persist and if so for how long. On the other hand, it is not at all clear that DNA damage can represent a lethal injury to a nonproliferating cell.

The present report uses a nonproliferating culture of rat hepatocytes to examine the relationship between the accumulation of DNA single-strand breaks and the killing of the cells by an oxidative stress. DNA single-strand breaks are readily detected following exposure of the cultured hepatocytes to TBHP or to hydrogen peroxide. However, cell killing can be prevented for extended times without any affect on the accumulation of single-strand breaks in DNA.

Materials and Methods

Male Sprague Dawley rats weighing 150–200 g (Charles River Breeding Laboratory, Wilmington, MA) were given food ad libitum and were fasted overnight before use. Isolated hepatocytes were prepared by collagenase perfusion, according to the method of Seglen (13). 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, at a density of 1.33 × 10^6 cells/flask, in 3 ml of Williams' E medium or 75-cm^2 flasks, at a density of 4 × 10^6 cells/flask, in 9 ml of medium. The medium contained 10 IU/ml penicillin, 10 μg/ml streptomycin, 0.05 mg/ml gentamicin, 0.02 units/ml insulin, and 10% heat-inactivated (55° for 15 min) fetal calf serum. After incubation for 2 hr at 37° in an atmosphere of 5% CO_2/95% air, the cultures were washed twice with a prewarmed HEPES buffer (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, and 2.4 mM HEPES, pH 7.4) to remove unattached dead cells. Complete Williams' E was replaced, and the cells were incubated for 24 hr. The cultures were then washed and incubated in Williams' E minus fetal calf serum with the additions indicated in the text. TBHP (Sigma Chemical Co., St. Louis, MO) and menadione (Sigma) were dissolved in serum-free Williams' E medium. Glucose oxidase (Sigma) was dissolved in distilled H_2O. Deferoxamine (Ciba Pharmaceutical Co., Summit, NJ) was dissolved in 0.9% NaCl and was added to the cultures at a final concentration of 20 mM. DPPD (Eastman Chemical Co., Rochester, NY) was dissolved in dimethyl sulfoxide and was added to the cultures at a final concentration of 1 μM (0.5% dimethyl sulfoxide). BHT (Sigma) was dissolved in dimethyl sulfoxide and was added to the cultures at a final concentration of 1 mM (0.5% dimethyl sulfoxide). KTBA (Sigma) was dissolved in 0.9% NaCl and was added to the culture medium to give a concentration of 40 mM. The viability of the cells was determined by the release of lactate dehydrogenase into the culture medium (14). The number of dead cells determined by lactate dehydrogenase release agreed exactly with the number of cells that take up trypan blue.

For experiments conducted in buffers with a different pH, the buffers contained 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 6.1 mM HEPES, 2 mM Tris, and 1.8 mM $CaCl_2$. At either pH 6.0 or 7.4, this buffer had no effect on the viability of control cells over the time courses studied nor did it interfere with the measurement of LDH activity.

DNA single-strand breaks were measured by the fluorometric analysis of the rate of DNA unwinding, according to the method of Birnboim and Jevcak (15). The cells were washed with a HEPES buffer (4°), scraped into 9 ml of the same buffer, collected by centrifugation, and resuspended in 650 μl of a solution containing 0.25 mM mesoinositol, 10 mM sodium phosphate, and 1 mM $MgCl_2$ (pH 7.2). The cells were distributed in 200-μl aliquots into three tubes (T, to measure total double-stranded DNA; P, to measure double-stranded DNA after unwinding in alkali; and B, to measure background fluorescence). Two hundred microliters of a solution containing 9 M urea, 10 mM NaOH, 2.5 mM cyclohexanediaminetetraacetate, and 0.1% sodium dodecyl sulfate were added to each tube. The samples were incubated at 0° for 10 min to lyse the cells and disrupt the chromatin. A 400-μl aliquot of a solution that was 1 M glucose and 14 mM mercaptoethanol was added to the T tubes to prevent any DNA unwinding. Then, 200 μl of a solution that was 0.45 volumes of the urea solution in 0.20 N NaOH was added to every tube. The DNA was allowed to unwind in these alkaline solutions for 30 min at 0°, followed by 15 min at 15°. Before the incubation at 15°, the B tubes were sonicated for 3 sec to allow complete denaturation of the DNA in these tubes. The unwinding was stopped with the addition of the glucose-mercaptoethanol solution to the P and B tubes, and the samples were placed in an ice bucket. Ethidium bromide (6.7 μg/ml in 13.3 mM NaOH) was then added to all the tubes, and the fluorescence of the samples was measured in a Perkin Elmer LS-5 spectrofluorometer (emission, 590 nm; excitation, 520 nm). The ratio of total double-stranded DNA fluorescence to background (fluorescence arising from other cellular sources) was between 1.8 and 2.1. The per cent double-stranded DNA was calculated as $(P - B)/(T - B) \times 100$. The assay was always performed under dim light.

All statistical analyses were performed using the Student's t test.

Results

DNA strand breaks and the toxicity of TBHP. Single-strand breaks in DNA were readily detected by monitoring the rate of the unwinding of double-stranded DNA when crude hepatocyte lysates were exposed to alkali (15). The decrease in the fluorescence of ethidium bromide bound to double-stranded DNA was used to monitor the rate of unwinding of hepatocyte DNA. Fig. 1 shows that exposure of cultured hepatocytes to 0.75 mM TBHP for 30 min produced single-strand breaks in DNA. This is indicated by the greater rate of unwinding of the DNA from the TBHP-treated hepatocytes, as opposed to con-

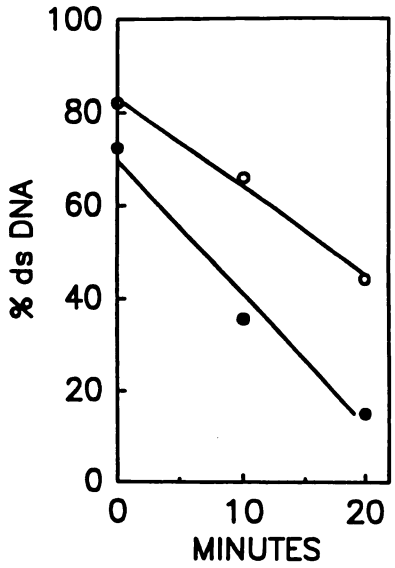

Fig. 1. Linearity of DNA unwinding in alkali. Cells were treated for 30 min in the presence (●) or absence (○) of 0.75 mM TBHP and were then kept in alkali at 0° for 30 min. Time indicates duration beyond this initial incubation, during which the hepatocyte lysates were incubated at 15°. Values are means of duplicates and are representative of several experiments.

trol cells, following exposure of crude lysates to alkali. The unwinding in Fig. 1 is linear in both cases over the 20-min time course of the incubation at 15°, a result that allowed subsequent data to be expressed as the extent of unwinding after 15 min. Thus, the data reported below are based on the extent of unwinding at one time point, namely 15 min. Nevertheless, for each separate experiment, the unwinding of the controls was always shown to be linear and triplicate samples agreed within 10%. However, between experiments there was variation in the extent of unwinding of control samples after 15 min. This variation is attributable to slight differences in technique between individuals and to subtle variations in the pH of the assay buffers.

Fig. 2 (*left*) illustrates the time course of the accumulation of single-strand breaks in DNA following exposure of the hepatocytes to 0.75 mM TBHP. Single-strand breaks were present within 5 min. Maximum DNA damage was observed within 15 min. Importantly, with 0.75 mM TBHP, cell killing is first detected after 30 min (see Fig. 3). Thus, the presence of single-strand breaks in DNA cannot be attributed simply to the death of the hepatocytes. The nuclear enzyme poly(ADP-ribose) polymerase is increased in response to DNA strand breaks (16–20). The activity of this enzyme increased after treatment of the cells with TBHP, and the time course of this increase was similar to that of the accumulation of single-strand breaks illustrated in Fig. 2, *left* (data not shown).

Fig. 2 (*right*) details the effect of increasing concentrations of TBHP on the accumulation of DNA single-strand breaks following a 30-min exposure to this toxin. The ability of a given concentration of TBHP to cause DNA single-strand breaks paralleled its ability to kill the cells after a 60-min exposure. With 0.25 mM TBHP, there were no single-strand breaks and no cell killing (data not shown). By contrast, 0.5 and 0.75 mM TBHP resulted in significant DNA damage at 30 min (Fig. 2, *right*) and 53 and 68% of the cells were dead at 60 min, respectively (data not shown).

Inhibition of glutathione reductase by BCNU sensitizes cultured hepatocytes to an oxidative stress (21). Table 1 documents that BCNU similarly sensitizes cultured hepatocytes to the toxicity of TBHP. In the absence of BCNU, 0.2 mM TBHP was not toxic to the cells. By contrast, after pretreatment with

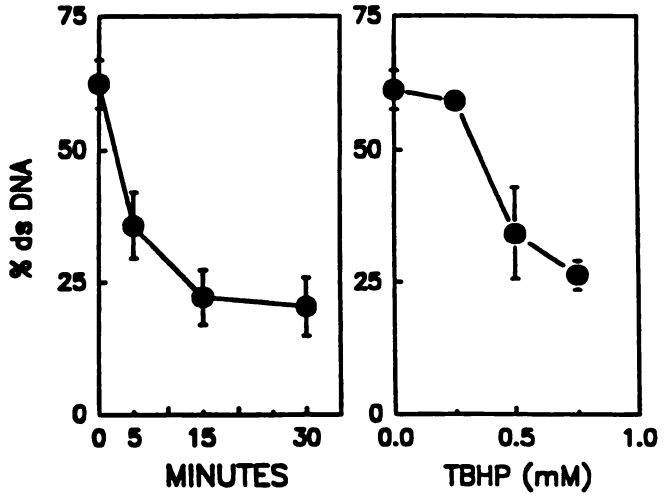

Fig. 2. *Left*, time course of 0.75 mM TBHP-induced DNA single-strand breaks. *Right*, TBHP dose response for DNA single-strand breaks. Hepatocytes were exposed to the listed concentrations for 30 min.

TABLE 1

Effect of BCNU on the accumulation of DNA single-strand breaks and cell killing with TBHP

Cultured hepatocytes were exposed to 0.2 mM TBHP for 30 min (for DNA damage) or 60 min (for lactate dehydrogenase release) with or without a 15-min pretreatment with 0.3 mM BCNU. The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	63.4 ± 6.1	1 ± 1
TBHP	68.7 ± 3.6	4 ± 1
BCNU alone	58.4 ± 4.6	2 ± 1
TBHP + BCNU	32.9 ± 10*	42 ± 5

* Significantly different from control, $p < 0.01$.

TABLE 2

Effect of deferoxamine pretreatment on the accumulation of DNA single-strand breaks and cell killing with TBHP

Cultured hepatocytes were treated with 20 mM deferoxamine for 1 hr, washed, placed in medium containing serum with no further addition, 1 mM $FeSO_4$, or 0.5 mM $FeCl_3$, and then incubated with or without 0.75 mM TBHP for 30 min (for DNA damage) or 60 min (for lactate dehydrogenase release). The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	71.7 ± 3.8	1 ± 1
TBHP alone	44.4 ± 6.6*	58 ± 4
Deferoxamine alone	70.0 ± 4.2	2 ± 1
TBHP + deferoxamine	69.4 ± 4.5	4 ± 1
$FeSO_4$ alone	68.9 ± 6.0	2 ± 1
TBHP + deferoxamine + $FeSO_4$	45.1 ± 2.6*	44 ± 3
$FeCl_3$ alone	67.0 ± 3.5	2 ± 1
TBHP + deferoxamine + $FeCl_3$	42.5 ± 3.9*	52 ± 4

* Significantly different from control, $p < 0.01$.

BCNU, 42% of the hepatocytes were killed by this same dose. Table 1 also shows that 0.2 mM TBHP produced no single-strand breaks after a 30-min exposure in the absence of the pretreatment with BCNU. By contrast, single-strand breaks were readily detected with 0.2 mM TBHP after a pretreatment with BCNU.

The killing of cultured hepatocytes by TBHP and the accumulation of single-strand breaks in DNA are dependent on a cellular source of ferric iron. Table 2 shows that pretreatment of the cells with the ferric iron chelator deferoxamine prevented the DNA single-strand breaks caused by 0.75 mM TBHP in parallel with the prevention of the cell killing. Treatment with deferoxamine alone produced no DNA damage or cell killing. Addition of 0.5 mM $FeCl_3$ to the cultures restored the sensitivity of the cells to TBHP, as measured by an accumulation of single-strand breaks and the death of the hepatocytes (Table 2). A similar result was obtained with $FeSO_4$ (Table 2).

Radical scavengers also protect the cultured hepatocytes from the lethal injury produced by TBHP. Table 3 indicates that the radical scavenger KTBA prevented the cell killing produced by a 60-min exposure to 0.75 mM TBHP. KTBA substantially reduced the evidence of DNA damage under the same conditions.

Dissociation of DNA damage from the cell killing by TBHP. TBHP kills cultured hepatocytes by a mechanism that is accompanied by the peroxidation of cellular lipids (22, 23). Accordingly, the killing of cultured hepatocytes by TBHP can be prevented by antioxidants (22). Importantly, with lower concentrations of TBHP the action of an antioxidant such as

TABLE 3

Effect of KTBA on the accumulation of DNA single-strand breaks and cell killing with TBHP

Cultured hepatocytes were exposed to 0.75 mM TBHP for 30 min (for DNA damage) or 60 min (for lactate dehydrogenase release) in the presence or absence of 40 mM KTBA. The results are the mean ± standard deviation of the determinations of three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	51.9 ± 5.0	1 ± 1
TBHP	5.6 ± 2.9[a]	67 ± 4
KTBA alone	55.0 ± 7.5	2 ± 1
TBHP + KTBA	23.4 ± 8.1[a,b]	3 ± 1

[a] Significantly different from control, $p < 0.01$.
[b] Significantly different from TBHP, $p < 0.01$.

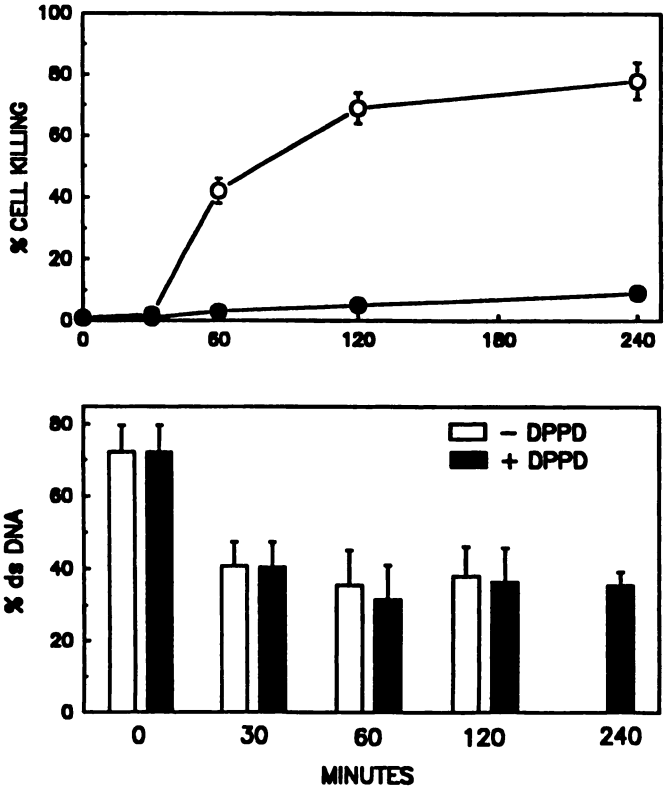

Fig. 3. Effect of DPPD on TBHP-induced DNA single-strand breaks. Hepatocytes were exposed to 0.50 mM TBHP for the times indicated in the presence or absence of 1 μM DPPD. *Top*, percentage of cell killing during these exposures. ○, Minus DPPD; ●, plus DPPD. *Bottom*, DNA single-strand break accumulation from these exposures. □, minus DPPD; ■, plus DPPD. Values are the mean percentage of double-stranded DNA ± standard deviation for three or four determinations. All values for percentages of double-stranded DNA at 30 min and beyond are significantly different from control, $p < 0.01$.

DPPD does not represent a delaying of the time of cell killing (22). Fig. 3 (*top*) shows that DPPD prevented the cell killing produced by 0.50 mM TBHP not just at 1 hr but over the entire 4-hr time course of the experiment. By contrast, DPPD had no effect on the extent of the DNA damage over the same 4-hr time course (Fig. 3, *lower*). Thus, in the presence of DPPD, there was a dissociation of the cell killing by TBHP from the production of DNA damage.

Another antioxidant, BHT, similarly prevented the toxicity of TBHP without preventing the accumulation of single-strand breaks. Table 4 shows that 0.75 mM TBHP killed 82% of the hepatocytes and resulted in substantial accumulation of single-strand breaks. The addition of 1 mM BHT reduced the number of dead cells to less than 10% of the total. However, there was no significant reduction in the extent of DNA damage in the presence of BHT.

Finally, acidification of the cell culture medium also protects hepatocytes from the toxicity of TBHP (24). Fig. 4 (*top*) indicates that exposure to 0.50 mM TBHP for 4 hr in a culture medium of pH 6.0 resulted in essentially complete prevention of the substantial cell killing that occurred in the same medium at pH 7.4. However, there was no difference in the extent of DNA damage at pH 6.0 compared with pH 7.4 (Fig. 4, *lower*), a result that again shows dissociation of cell killing from DNA damage. Acidification of the medium in the absence of TBHP had no effect on either cell viability or on the integrity of the DNA.

DNA strand breaks and the toxicity of hydrogen peroxide. The generation of hydrogen peroxide in the culture medium by glucose oxidase is another useful model with which to study oxidative cell injury (25). Fig. 5 (*left*) shows that increasing concentrations of glucose oxidase produced increasing DNA single-strand breaks after a 30-min exposure to the enzyme. Fig. 5 (*right*) illustrates the time course of the accumulation of DNA single-strand breaks after exposure of cells to 2 units/ml glucose oxidase. DNA single-strand breaks were evident within 5 min. No further DNA damage accumulated beyond 15 min. The 50% cell killing produced by 2 units/ml glucose oxidase was not observed until 45 min (data not shown).

The killing of cultured hepatocytes by H_2O_2 is also dependent upon a cellular source of ferric iron (26). Similarly, the appearance of DNA single-strand breaks in cells treated with glucose oxidase depended on a cellular source of iron (Table 5). Pretreatment of the hepatocytes with deferoxamine prevented the DNA single-strand breaks produced by 2 units/ml glucose oxidase. Again, the addition of $FeCl_3$ to the medium following a pretreatment with deferoxamine restored the sensitivity of the cells to glucose oxidase, with resultant cell killing and an accumulation of DNA single-strand breaks. The addition of $FeSO_4$ to the medium produced a similar effect.

Dissociation of DNA damage from the cell killing by hydrogen peroxide. Acidification of the cell culture medium was used to dissociate the cell killing produced by H_2O_2 from the accumulation of single-strand breaks. Table 6 shows that exposure of the hepatocytes to 1 unit/ml glucose oxidase in culture medium at pH 6.0 prevented, for the entire 4-hr time course of the experiment, the cell killing that occurred in the same medium at pH 7.4. Nevertheless, the accumulation of

TABLE 4

Effect of BHT on the accumulation of DNA single-strand breaks and cell killing produced by TBHP

Cultured hepatocytes were exposed to 0.75 mM TBHP for 30 min (for DNA damage) or 60 min (for lactate dehydrogenase release) in the presence or absence of 1 mM BHT. The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	70.6 ± 8.8	1 ± 1
TBHP	44.7 ± 7.6[a]	82 ± 7
BHT alone	67.4 ± 5.0	4 ± 2
TBHP + BHT	34.6 ± 3.3[a]	8 ± 2

[a] Significantly different from control, $p < 0.01$.

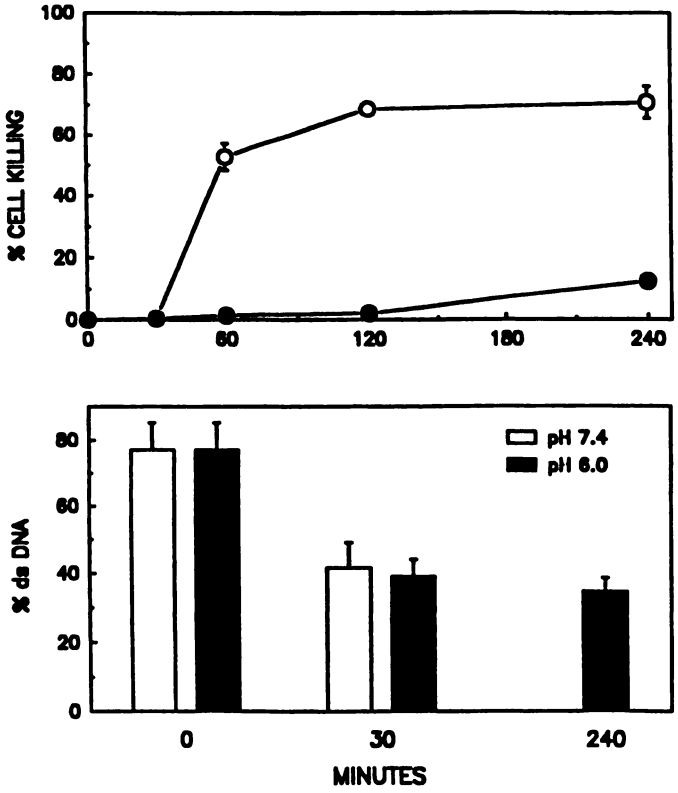

Fig. 4. Effect of extracellular acidosis on TBHP-induced DNA single-strand breaks. Hepatocytes were exposed to 0.50 mM TBHP for the times indicated in a culture medium at pH 7.4 or 6.0. *Top*, percentage of cell killing during these exposures. ○, pH 7.4; ●, pH 6.0. *Bottom*, DNA single-strand break accumulation from these exposures. □, pH 7.4; ■, pH 6.0. Values are the mean percentages of double-stranded DNA ± standard deviation for three or four determinations. All values for percentage of double-stranded DNA at 30 min and beyond are significantly different from control, $p < 0.01$.

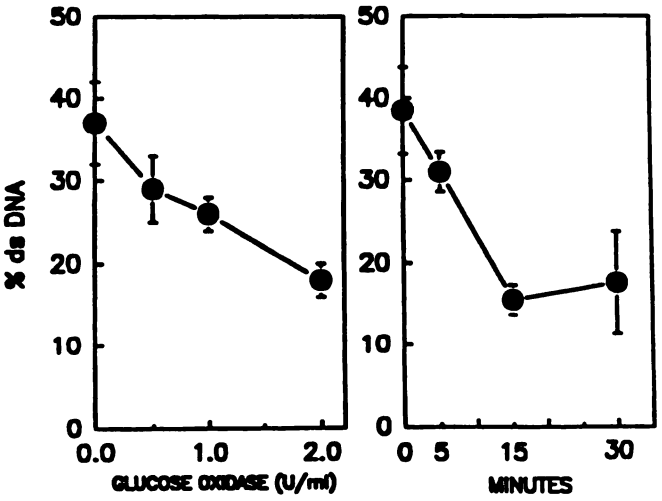

Fig. 5. Glucose oxidase dose response on DNA single-strand breaks. *Left*, hepatocytes were exposed to varying concentrations of glucose oxidase for 30 min. Glucose oxidase time course of DNA single-strand breaks. *Right*, hepatocytes were exposed to 2 units/ml glucose oxidase for the various times. Values are the mean percentage of double-stranded DNA ± standard deviation for three or four determinations.

TABLE 5

Effect of deferoxamine on the accumulation of DNA single-strand breaks and cell killing produced by glucose oxidase

Cultured hepatocytes were treated with 20 mM deferoxamine, washed, placed in medium containing serum with no further addition, 1 mM $FeSO_4$, or 0.5 mM $FeCl_3$, and then incubated with or without 2 units/ml glucose oxidase for 30 min (for DNA damage) or 60 min (for lactate dehydrogenase release). The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	77.1 ± 9.4	1 ± 1
Glucose oxidase	53.5 ± 8.0[a]	47 ± 4
Deferoxamine alone	74.7 ± 6.2	1 ± 1
Glucose oxidase + deferoxamine	71.7 ± 9.3	2 ± 1
$FeSO_4$ alone	75.0 ± 5.0	1 ± 1
Glucose oxidase + deferoxamine + $FeSO_4$	45.6 ± 7.4[a]	44 ± 5
$FeCl_3$ alone	76.0 ± 4.0	2 ± 1
Glucose oxidase + deferoxamine + $FeCl_3$	48.4 ± 3.6[a]	49 ± 3

[a] Significantly different from control, $p < 0.01$.

TABLE 6

Effect of extracellular acidosis on the accumulation of DNA strand breaks and cell killing with glucose oxidase

Cultured hepatocytes were exposed to 1 unit/ml glucose oxidase for 30 min (for DNA damage) or 1 or 4 hr (for lactate dehydrogenase release) in a buffer at either pH 7.4 or pH 6.0. The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	pH	Double-Stranded DNA	Cell Killing	
			1 hr	4 hr
		%		
Control	7.4	52.4 ± 5.5	2 ± 1	4 ± 6
Glucose oxidase	7.4	34.5 ± 4.9[a]	37 ± 3	61 ± 6
Control	6.0	55.7 ± 6.8	1 ± 1	3 ± 2
Glucose oxidase	6.0	37.0 ± 2.6[a]	2 ± 1	7 ± 2[b]

[a] Significantly different from control, $p < 0.001$.
[b] The percentage of double-stranded DNA after 4 hr was 33.3 ± 5.2.

DNA single-strand breaks remained the same for the entire 4 hr, regardless of whether the cells were exposed to H_2O_2 at pH 6.0 or at pH 7.4.

DNA strand breaks and the toxicity of menadione. The redox cycling of menadione exerts an oxidative stress on cultured hepatocytes as a result of the generation of hydrogen peroxide. Quinones like menadione undergo a one-electron reduction to yield the corresponding semiquinone radical. Oxidation of such semiquinone radicals by molecular oxygen produces superoxide anions, with regeneration of the parent compound. The enzymatic or spontaneous dismutation of superoxide anions yields H_2O_2.

The exposure of cultured hepatocytes to menadione is accompanied by evidence of DNA damage (27, 28). Fig. 6 (*left*) shows the accumulation of DNA single-strand breaks following exposure of the hepatocytes to increasing concentrations of menadione for 30 min. Increasing concentrations of menadione resulted in greater DNA damage. Fig. 6 (*right*) details the time course of the accumulation of single-strand breaks following exposure of the hepatocytes to 500 μM menadione. Strand breaks were not evident until 30 min, and they did not increase after that time. Importantly, the cell killing produced by 500 μM menadione was not evident until at least 2 hr after exposure. Thus, the delay in the appearance of DNA damage is paralleled by the longer time course of the evolution of the irreversible injury. Nevertheless, DNA damage again clearly precedes the death of the cells.

The toxicity of menadione depends on a cellular source of

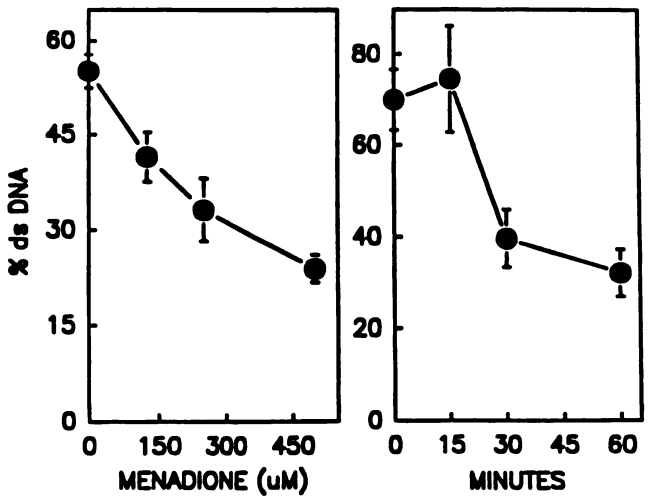

Fig. 6. Effect of menadione on DNA single-strand breaks. *Left*, hepatocytes were treated with varying concentrations of menadione for 30 min. *Right*, time course of menadione-induced DNA single-strand breaks. Hepatocytes were exposed to 500 mM menadione for the various times. Values are the mean percentage of double-stranded DNA ± standard deviation for three or four determinations.

TABLE 7

Effect of deferoxamine on the accumulation of DNA single-strand breaks and cell killing produced by menadione

Cultured hepatocytes were pretreated with 20 mM deferoxamine for 60 min, washed, and then incubated with or without 0.5 mM menadione for 30 min (for DNA damage) or 3 hr (for lactate dehydrogenase release). The results are the mean ± standard deviation of the determinations on three or four separate cultures.

Treatment	Double-Stranded DNA	Dead Cells
	%	
Control	73.0 ± 9.2	4 ± 1
TBHP	57.8 ± 1.2[a]	29 ± 3
Deferoxamine alone	74.1 ± 2.7	5 ± 2
TBHP + deferoxamine	71.1 ± 4.0	8 ± 2

[a] Significantly different from control, $p < 0.02$.

ferric iron (26). Table 7 indicates that exposure of the hepatocytes to 500 μM menadione for 3 hr resulted in 30% cell killing, whereas cells pretreated with deferoxamine were protected from this cell killing. Again, the preventive effect of deferoxamine on cell killing was accompanied by a suppression of the accumulation of DNA single-strand breaks.

Acidification of the culture medium allowed a dissociation of cell killing with menadione from the accumulation of DNA single-strand breaks (Table 8). Exposure of the hepatocytes to 250 μM menadione in a medium at pH 6.0 prevented, over the entire 4-hr time course of the experiment, the cell killing that occurred in the same medium at pH 7.4. By contrast, the extent of DNA damage produced was the same at each pH. Thus, protection of the hepatocytes was again achieved without any change in the extent of DNA damage.

Discussion

The present study explored conditions that affect that DNA damage produced by an oxidative stress in primary cultures of rat hepatocytes. TBHP, glucose oxidase, and menadione were used to impose an oxidative stress on the hepatocyte. In each case similar effects on DNA damage and cell killing were observed.

TABLE 8

Effect of extracellular acidosis on the accumulation of DNA strand breaks produced by menadione

Cultured hepatocytes were exposed to 250 μM menadione for 1 hr (for DNA damage) or 4 hr (for cell killing) in a medium at either pH 7.4 or pH 6.0. For exposures to menadione in the medium at pH 6.0, DNA damage was assessed at both 1 and 4 hr. Values are the mean ± standard deviation of three cultures.

Treatment	pH	Double-Stranded DNA	Dead Cells
		%	
Control	7.4	72.5 ± 6.7	5.8 ± 1.9
Menadione	7.4	37.3 ± 7.5[a]	53.9 ± 2.0
Control	6.0	68.3 ± 4.2	2.6 ± 0.6
Menadione	6.0	35.6 ± 7.8[a]	6.2 ± 1.5[b]

[a] Significantly different from control, $p < 0.01$.
[b] The percentage of double-stranded DNA after 4 hr was 29.9 ± 6.1.

Many features of the data presented above emphasize the close relationship between DNA damage and cell killing that has been emphasized previously (1, 3, 5–9). With each of the hazards studied, there was a parallel dose-response relationship between the accumulation of DNA single-strand breaks and the extent of cell killing. In each case the appearance of DNA damage preceded the loss of cell viability, a result that indicates that the strand breaks are not simply the result of the death of the cells. Inhibition of glutathione reductase by BCNU, known to potentiate the sensitivity of cultured hepatocytes to an oxidative stress, similarly sensitized the cells to the induction of DNA damage.

In each case studied, both the cell killing and the DNA single-strand breaks required a cellular source of iron. The pretreatment of the cells with the ferric iron chelator deferoxamine protected the hepatocytes from both the accumulation of single-strand breaks and the cell killing. Furthermore, the addition of iron to the medium restored the sensitivity of the cells to both phenomena. In a number of other models of oxidative stress, lethal cell injury (1, 5, 29, 30) and DNA damage (1, 5, 31, 32) were similarly shown to depend on a source of ferric iron.

The requirement for a cellular source of ferric iron presumably relates to the formation of a potent oxidizing species from hydrogen peroxide or TBHP, such as the hydroxyl or *tert*-butyl alkoxyl radical (4). Thus, by chelating iron, deferoxamine prevents the formation of the agent that is actually responsible for the initiation of the DNA damage that results in the appearance of a single-strand break. The formation of such an oxidizing species also explains the ability of a radical scavenger such as KTBA to reduce the extent of the DNA damage.

The hydroxyl and *tert*-butyl alkoxyl radical are very reactive species that are capable of interacting with cellular constituents other than DNA (33). Furthermore, an effect on a cellular constituent other than DNA may be equally if not more important than DNA damage in the pathogenesis of lethal cell injury. Thus, the protective effect of iron chelation against the lethal cell injury occurring with an oxidative stress cannot necessarily be attributed to the protective effect against the accumulation of single-strand breaks in DNA. Clearly, the other chemical effects of the hydroxyl or alkoxyl radical are likely to be prevented by iron chelation, as well as by radical scavengers. Thus, in order to assess specifically the role of DNA damage in the cell killing by an oxidative stress, it was necessary to manipulate the sensitivity of the hepatocytes to activated oxygen species by means that act on events that occur after the formation of either the hydroxyl or *tert*-butyl alkoxyl radical.

The peroxidation of cellular lipids can be initiated by the hydroxyl or *tert*-butyl alkoxyl radical. Furthermore, the peroxidation of cellular lipids that occurs during the course of the intoxication of hepatocytes with either hydrogen peroxide or TBHP is related to the killing of the cells. This conclusion is illustrated by the action of the protective agent DPPD, an antioxidant that can scavenge lipid radicals.

DPPD prevents both the peroxidation of lipids and the death of the cells. Importantly, DPPD acts to prevent the peroxidation of lipids at a point distal to the formation of either hydroxyl or alkoxyl radicals (22). That is, DPPD does not act by scavenging these radicals but rather by reacting with the lipid radicals in the membranes of the cell (22). Thus, the hepatocytes are protected from a lethal injury at a step that occurs after the formation of radicals that damage DNA. Thus, DNA damage still occurred at the same time that DPPD protected from the cell killing (Fig. 3).

The acidification of the culture medium also protected the hepatocytes from a lethal injury without an effect on the extent of DNA damage (Fig. 4). Interestingly, extracellular acidosis does not prevent the lipid peroxidation induced by TBHP (24). Most likely, the acidosis acts to stabilize peroxidized membranes from a loss of functional integrity. Accordingly, the DNA from cells exposed to an oxidative stress in an acidified medium was damaged to the same extent, despite the fact that there was essentially no cell killing over the entire 4-hr time course of the experiment.

An alternative interpretation of the effects of both DPPD and an extracellular acidosis would argue that both manipulations act to prevent some mechanism that couples DNA damage to lethal cell injury. However, such a conclusion would have to postulate that the presumed coupling mechanism acts to initiate lipid peroxidation. Evidence for such a mechanism is lacking in hepatocytes. Interestingly, a mechanism coupling single-strand breaks in DNA to lethal cell injury has been proposed to operate in the killing of P388D_1 cells exposed to hydrogen peroxide (4). A deficiency of pyridine nucleotides following the activation of poly(ADP-ribose) polymerase was suggested to result in loss of ATP and then of viability. Whereas ATP depletion by other mechanisms did not lead to irreversible cell injury in P388D_1 cells (34), a depletion of pyridine nucleotides upon activation of DNA repair processes may act to potentiate lethal cell injury by another mechanism. If such a mechanism is operative under the conditions of the oxidative cell injury studied here, it must still relate to the initiation or spread of lipid peroxidation.

An important conclusion emerges from this discussion. Attempts to establish the role that DNA damage plays in oxidative cell killing based on manipulations that prevent the formation of an ultimate toxic oxygen species, namely the chelation of iron or the scavenging of radicals, may be misleading. Such an analysis must be accompanied by an attempt to distinguish among the different lesions produced by these radicals. When such a concern is observed, our data show that damage to DNA can be dissociated from the mechanisms of the oxidative killing of such nonproliferating cells as cultured hepatocytes. Stated differently, our data indicate that the killing of cultured hepatocytes by an acute oxidative stress does not necessarily result from the evident damage to the DNA.

The relevance of this conclusion to the oxidative killing of proliferating cells needs to be discussed. Two points should be emphasized. Firstly, proliferating cells may be susceptible to mechanisms of cell killing that are not operative in nonproliferating cells. There are data to indicate that proliferating cells that are deficient in the repair of DNA damage are more sensitive to the lethal injury produced by an oxidative stress (10–12). Oxidative DNA damage in a proliferating cell may initiate a sequence of biochemical events that eventuates in the loss of viability. Secondly, DNA damage in a proliferating cell may directly interfere with replication without necessarily compromising, at least initially, viability. Viability assays based on the ability of the injured cells to replicate would score such a cell as nonviable. Thus, our data would imply that there may be an important distinction between the static versus lytic consequences of DNA damage in a proliferating cell population.

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