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Autophagic Degradation of Protein Generates a Pool of Ferric Iron Required for the Killing of Cultured Hepatocytes by an Oxidative Stress

ISAO SAKAIDA,[1] MARLENE E. KYLE,[2] and JOHN L. FARBER
Department of Pathology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
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SUMMARY

Pretreatment of cultured hepatocytes with the ferric iron chelator deferoxamine prevents the killing of the cells by *tert*-butyl hydroperoxide (TBHP). Incubation of the deferoxamine-pretreated hepatocytes in a serum-free medium containing only 0.25 nM iron restored the sensitivity of the cells to TBHP within 4 to 6 hr. An amino acid-free medium accelerated the restoration of sensitivity in parallel with an enhanced rate of degradation of ^{14}C-prelabeled protein. By contrast, inhibitors of the autophagic degradation of protein, including chymostatin, 3-methyladenine, benzyl alcohol, colchicine, oligomycin, and methylamine, inhibited the restoration of sensitivity of deferoxamine-treated hepatocytes to TBHP in parallel with their inhibition of protein degradation. With chymostatin, 3-methyladenine, benzyl alcohol, and colchicine, there was a parallel dose dependency of both the inhibition of protein turnover and the inhibition of the restoration of sensitivity to TBHP. Ascorbic acid, known to specifically retard the autophagic degradation of ferritin, inhibited the restoration of sensitivity to TBHP without effect on the general rate of protein turnover. None of the agents studied had any protective effect on the toxicity of TBHP for hepatocytes that were not pretreated with deferoxamine. These data indicate that the autophagic degradation of protein generates a pool of ferric iron required for the killing of cultured hepatocytes by TBHP.

The killing of cultured rat hepatocytes by an oxidative stress depends on a cellular source of ferric iron (1). Pretreatment of hepatocytes with deferoxamine, a ferric iron chelator, reduced the cell killing by the hydrogen peroxide that was generated in the culture medium by glucose oxidase or within the hepatocytes by the metabolism of menadione (1). The sensitivity to hydrogen peroxide was restored by the addition of either ferric or ferrous iron to the culture medium (1). Superoxide dismutase reduced the killing by H_2O_2 of either native hepatocytes or hepatocytes that had been pretreated with deferoxamine and then given ferric, but not ferrous, iron (1). These data imply that the toxicity of H_2O_2 relates to the formation of a potent oxidizing species, such as the hydroxyl radical or an equivalently reactive species, by an iron-catalyzed Haber-Weiss reaction. In particular, a cellular pool of ferric iron is first reduced to ferrous iron by superoxide anions. In turn, hydrogen peroxide is reduced by this ferrous iron to give the ultimate toxin.

This conclusion left unresolved a number of issues, in particular, the nature of the cellular source of the iron required for cell killing, its location within the hepatocyte, and the mechanism that generates it. The lysosomotropic amines methylamine and chloroquine prevented the killing of cultured hepatocytes by H_2O_2 (2). Importantly, maximum protection required several hours of preincubation with either amine. The sensitivity of the hepatocytes to H_2O_2 was restored by incubating the cells for 4 hr in the absence of either amine before the treatment with H_2O_2 (2).

Elevation of the pH of acidic vacuolar compartments by lysosomotropic amines affects both the uptake (3) and release of iron (4) by hepatocytes. Iron-laden transferrin is taken up by endocytosis, and the subsequent release of iron occurs after acidification of the endocytic vesicles (3, 5–7). Acidification can occur either by the fusion of the vesicles with lysosomes or, alternatively, in a distinct prelyososomal vacuolar compartment. In addition, the mobilization of iron from ferritin may also involve the acidic vacuolar apparatus (4, 8). The autophagocytosis of cytosolic ferritin promotes iron release within lysosomes, due to the conjugate action of an acidic pH and hydrolytic enzymes. Thus, protection by lysosomotropic amines likely involves an interference with the formation of a deferoxamine-chelatable pool of ferric iron formed as a result of either the entrance of iron into the hepatocyte or its mobilization from intracellular ferritin stores.

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[1] On leave from the First Department of Internal Medicine, Yamaguchi University School of Medicine, Ube 755, Japan.
[2] Recipient of an individual National Research Service Award (1F32 ESGM 054180).

ABBREVIATIONS: TBHP, *tert*-butyl hydroperoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The present report details an experimental model with which we have further explored the mechanisms generating the ferric iron pool that is required for the killing of cultured hepatocytes by an oxidative stress. Hepatocytes that were depleted of this critical pool of ferric iron by a pretreatment with deferoxamine recovered sensitivity to an oxidative stress, in this case TBHP, upon incubation in a deferoxamine-free medium for 4–6 hr. Manipulations that activated or inhibited the autophagic turnover of protein altered in parallel the rate of restoration of sensitivity to TBHP. These data indicate that the autophagic turnover of protein generates a pool of ferric iron that is required for the killing of hepatocytes by an oxidative stress.

Materials and Methods

Cell cultures and reagents. Male Sprague-Dawley rats (150–200 g) were obtained from Charles River Breeding Laboratories, Inc. All animals were fed *ad libitum* and fasted overnight before use. Hepatocytes were isolated by collagenase (Sigma) perfusion according to the method of Seglen (9). Yields of 3–5 × 10^8 cells/liver with 85–90% 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 Laboratories) containing 10 IU/ml penicillin, 10 μg/ml streptomycin, 0.5 mg/ml gentamicin, 0.02 unit/ml insulin, and 10% heat-inactivated (55° for 15 min) fetal calf serum (Hazelton Research Products, Lenexa, KS) (complete Williams' E). After 2 hr at 37° in an atmosphere of 5% CO_2/95% air, the cultures were rinsed twice with a prewarmed HEPES (Sigma) buffer, pH 7.4 (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, and 2.4 mM HEPES), to remove unattached dead cells. Complete Williams' E (5 ml) was replaced, and the cells were incubated for 24 hr. The cultures were then washed and incubated in Williams' E minus fetal calf serum, alone or with 20 mM deferoxamine (Ciba-Geigy) dissolved in 0.9% NaCl, for 60 min. The cultures were washed twice with prewarmed HEPES buffer and returned to the varying conditions detailed in the text. Oligomycin (Sigma), chymostatin (Sigma), and benzyl alcohol (Sigma) were dissolved in dimethyl sulfoxide (the final concentration of dimethyl sulfoxide was always 0.5%). Colchicine (Sigma), methylamine (Sigma), ferric chloride (Fisher), and ascorbic acid (Fisher) were dissolved in 0.9% NaCl. 3-Methyladenine (Fluka) and TBHP (Sigma) were dissolved directly in the medium. The viability of the hepatocytes was measured 1 hr following exposure to TBHP, by the release of lactate dehydrogenase into the medium, as described previously (10).

Measurement of protein turnover. Protein turnover was measured by a modification of the methods of Seglen *et al.* (11) and Hopgood *et al.* (12). Two hours after the cells were plated, hepatocyte proteins were labeled for 24 hr in complete Williams' E medium containing 0.5 μCi of [^{14}C]valine (285 mCi/mmol; Amersham). After two washes with HEPES buffer, the labeling medium was then replaced by Williams' E medium with 2 mM valine without serum. After 1 hr, the cells were washed three times with HEPES buffer and incubated in a Krebs-Ringer bicarbonate buffer plus 10 mM glucose (13), containing antibiotics alone or with the varying agents as described in the text, for an additional 2.5 hr. Triton X-100 was then added to the cultures (final concentration of 0.5%). Hepatocyte proteins were precipitated by the addition of perchloric acid (final concentration, 10%). After 15 min of incubation at 0° and then centrifugation for 10 min at 5000 rpm (Dupont, RC5C), the radioactivity in the acid-soluble and -insoluble fractions was determined by liquid scintillation counting (11). The net release of [^{14}C]valine during the 2.5-hr incubation period was expressed as a percentage of the total initial protein radioactivity of the cell samples. All experiments were performed on triplicate cultures. All experiments were repeated three to five times.

Measurement of ferritin degradation. The degradation of ^{59}Fe-labeled ferritin was measured by modification of the method of White *et al.* (14). Two hours after the freshly isolated cells were initially plated, the cultures were washed and 1 μCi/ml [^{59}Fe]ferric chloride (1 mCi/ml; Amersham) was added to the fresh complete Williams' E medium. Twenty-four hours later, the cultures were washed four times with HEPES buffer and placed in serum-free Williams' E medium containing 20 mM deferoxamine. One hour later, the cells were washed and divided into two groups. One group was processed immediately by scraping the cells from the flasks into 5 ml of phosphate-buffered saline and disrupting them by sonication. The second group was reincubated in Krebs-Ringer bicarbonate buffer for 2.5 hr, after which time the cells were scraped and sonicated in 5 ml of phosphate-buffered saline. All samples were centrifuged at 10,000 × *g* for 30 min at 4° and the supernatants were incubated with rabbit anti-horse ferritin immunoglobulin (Dako) at 25° for 1 hr. Immunobead goat anti-rabbit immunoglobulin (Bio-Rad) was then added to the supernatant and incubated at 25° for an additional 2 hr. The samples were centrifuged at 3000 rpm for 10 min (Dupont, RC5C). The precipitate was washed three times with phosphate-buffered saline, and the radioactivity in the immunoprecipitate was determined in a γ scintillation counter (Beckman Gamma 5500B). Nonspecific binding, measured as the radioactivity precipitated by the addition of normal rabbit serum and Immunobead goat anti-rabbit immunoglobulin to the hepatocyte supernatants, was subtracted from each sample. The ability of rabbit anti-horse ferritin antibody to cross-react with rat ferritin was established by the Ouchterlony technique and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Results

Restoration of sensitivity to TBHP in deferoxamine-pretreated hepatocytes. TBHP has frequently been used to probe the mechanisms of cell injury by an acute oxidative stress (15–24). The killing of cultured hepatocytes by TBHP depends on a cellular source of ferric iron (24), and this iron pool can be readily depleted by treating the cells with deferoxamine, a ferric iron chelator.

Hepatocytes in culture for 24 hr were washed and exposed to 20 mM deferoxamine in serum-free medium. One hour later, the cells were washed again, placed in fresh serum-free medium (without insulin), and exposed to 0.3 mM TBHP. Without the deferoxamine pretreatment, TBHP killed 58% of the hepatocytes within 1 hr. By contrast, only 5% of the cells were killed following the pretreatment with deferoxamine.

The sensitivity to TBHP of cultured hepatocytes that were pretreated with deferoxamine was restored by incubating the cells for several hours before exposing them to the toxin. In the experiment illustrated in Fig. 1, the hepatocytes were washed, treated with deferoxamine for 1 hr, rewashed, and then placed in insulin-free Williams' E medium with or without 10% fetal calf serum. Then, 1, 2, 4, or 6 hr later, the ability of 0.3 mM TBHP to kill the cells was assessed. The data at each of the time points illustrated in Fig. 1 are expressed as the percentage of restoration of sensitivity of the hepatocytes to TBHP. Complete restoration of sensitivity indicates cell killing equivalent to that which occurred in cultures that were never pretreated with deferoxamine. Thus, at time 0, immediately following the treatment with deferoxamine, the percentage of restoration of sensitivity was 9% of native cells; that is, only 5% of the cells were killed by 0.3 mM TBHP, as opposed to 55% of the cells that were not pretreated with deferoxamine.

After 6 hr of incubation in Williams' E medium, the sensitivity of the hepatocytes to TBHP was completely restored. That is, treatment of these cells for 1 hr with 0.3 mM TBHP resulted in the death of 55% of the hepatocytes. The presence of serum had no effect on the rate of this restoration.

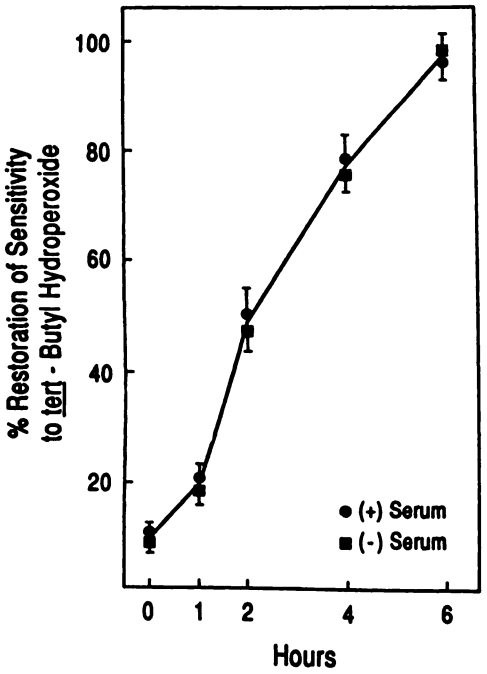

Fig. 1. Restoration of sensitivity to TBHP. Hepatocytes in culture for 24 hr were washed twice and placed in serum-free Williams' E medium containing 20 mM deferoxamine. After 1 hr, the cultures were washed again and placed in insulin-free Williams' E medium either with or without serum. At the times indicated, the cells were treated with 0.3 mM TBHP. The percentage of restoration was calculated as the ratio of dead cells after deferoxamine pretreatment to dead cells (58%) in untreated control cultures (TBHP treatment alone). The results represent the mean ± standard deviation of the determinations on three separate flasks.

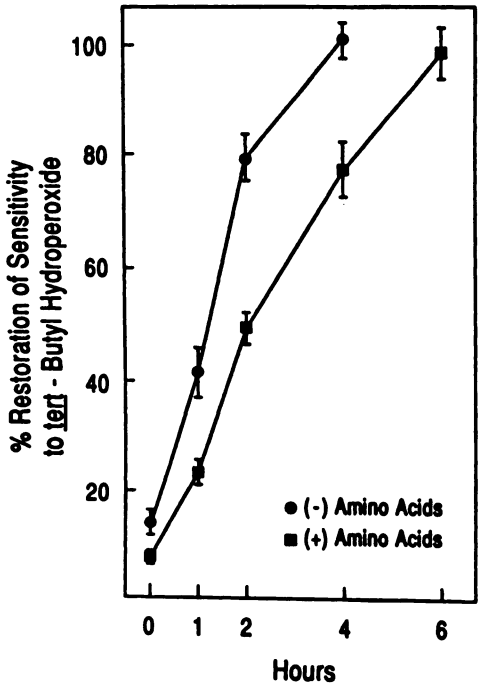

Fig. 2. The effect of amino acids on the rate of restoration of sensitivity to TBHP. Hepatocytes that had been pretreated with deferoxamine were placed in insulin-free Williams' E medium without serum or in an insulin-free Krebs-Ringer bicarbonate buffer (no amino acids). At the times indicated, the cells were treated with TBHP and viability was assessed 1 hr later. Complete restoration of sensitivity represents the killing by TBHP of 60% of the cells in Williams' E medium and 67% of the cells in Krebs-Ringer bicarbonate buffer. The results shown are the mean ± standard deviation of the determinations on three separate cultures.

The sensitivity to TBHP that resulted from incubations of 1–6 hr following the pretreatment with deferoxamine could again be removed by a second treatment with deferoxamine (data not shown). Thus, the initial loss of sensitivity and its restoration are related to a cellular pool of ferric iron that can be readily removed by treatment with deferoxamine.

In the absence of serum, the iron content of Williams' E medium is 0.25 nM. The addition of as much as 100 μM ferric chloride to Williams' E medium in the absence of serum had no effect on the rate of the restoration of sensitivity to TBHP shown in Fig. 1. Thus, it is unlikely that the iron content of the medium is a limiting factor in the rate of restoration of sensitivity of the hepatocytes.

Amino acid depletion accelerates restoration of sensitivity. The presence of amino acids in the culture medium affected the rate of restoration of sensitivity to TBHP. Following a pretreatment with deferoxamine, one group of cultures was placed in fresh serum-free Williams' E medium (without insulin). A second group was placed in a Krebs-Ringer bicarbonate buffer containing 10 mM glucose and antibiotics. The rate of restoration of sensitivity to TBHP in each case is illustrated in Fig. 2. Williams' E medium contains antioxidants that were not present in the Krebs-Ringer bicarbonate buffer. Accordingly, the hepatocytes in the latter medium were more sensitive to TBHP than were the same cells in Williams' E. Thus, 0.15 mM TBHP killed 67% of non-deferoxamine-pretreated cells in Krebs-Ringer bicarbonate buffer, whereas 0.3 mM TBHP killed 60% of the same cells incubated in Williams' E medium minus serum.

The data shown in Fig. 2 reflect the rate of restoration of the sensitivity of hepatocytes pretreated with deferoxamine to either 0.15 or 0.3 mM TBHP depending on whether the cells were incubated in Krebs-Ringer bicarbonate buffer or Williams' E medium, respectively. The hepatocytes in the Krebs-Ringer buffer recovered sensitivity to TBHP at a faster rate than did the same cells incubated in Williams' E medium. In Krebs-Ringer buffer, sensitivity to TBHP was completely restored within 4 hr, whereas it required 6 hr to achieve the same sensitivity in Williams' E medium. Importantly, the addition to the Krebs-Ringer bicarbonate buffer of a complement of amino acids equivalent to that in Williams' E medium slowed the rate of recovery to that seen with Williams' E medium (data not shown).

Amino acid starvation accelerates the autophagic turnover of proteins (25, 26). The accelerated restoration of sensitivity to TBHP of hepatocytes incubated in the Krebs-Ringer buffer (Fig. 2) was accompanied by enhanced protein degradation. Protein degradation was measured as the release of [^{14}C]valine from proteins prelabeled for 24 hr. The rate of protein turnover was 30% faster in hepatocytes incubated in Krebs-Ringer buffer (4.7 ± 0.1%/hr) as opposed to Williams' E medium (3.3 ± 0.2%/hr). The rate of protein degradation is similar to that reported previously in cultured rat hepatocytes (27).

Inhibition of autophagocytosis reduces the rate of restoration of sensitivity to TBHP. The autophagic degradation of protein can be inhibited by a variety of chemicals that interfere with the different steps of this process. A number of such inhibitors prevented the restoration of sensitivity to TBHP following a pretreatment with deferoxamine.

Chymostatin is a lysosomal protease inhibitor that inhibits

protein turnover in cultured hepatocytes (28). Fig. 3 illustrates the inhibitory effect of increasing concentrations of chymostatin both on the degradation of protein and on the restoration of sensitivity to TBHP. The effect on protein degradation was measured by adding increasing concentrations of chymostatin to cells in Krebs-Ringer bicarbonate buffer that had been prelabeled with [^{14}C]valine. After 2.5 hr, the percentage of inhibition of protein degradation was measured and the resulting data are shown in Fig. 3. Increasing concentrations of chymostatin resulted in a dose-dependent inhibition of protein degradation. Pretreatment of the hepatocytes with deferoxamine had no effect on the inhibition of protein degradation by chymostatin or the other agents discussed below.

Separate cultures were treated with deferoxamine, washed, incubated in Krebs-Ringer bicarbonate buffer for 2.5 hr with the same concentrations of chymostatin, and then treated with 0.15 mM TBHP. After 1 hr, the extent of cell killing was determined and is expressed in Fig. 3 as the percentage of inhibition of restoration of sensitivity to TBHP. In the absence of chymostatin, deferoxamine-treated cells recovered complete sensitivity to TBHP after the 2.5-hr incubation in Krebs-Ringer bicarbonate buffer. That is, the same number of cells (58% of the total) were killed by TBHP in cultures that were not treated with deferoxamine as in those treated with deferoxamine and incubated in its absence for 2.5 hr. Fig. 3 shows that increasing concentrations of chymostatin were associated with a dose-dependent inhibition of the restoration of sensitivity to TBHP. For example, 100 μg/ml chymostatin inhibited the restoration of sensitivity by 48%. That is, only 30% of the hepatocytes (versus 58% of the controls) were killed after the 2.5-hr incubation with this concentration of chymostatin. Importantly, 100 μg/ml chymostatin had no effect on the toxicity of TBHP in cultures that had not been pretreated with deferoxamine. That is, the effect of chymostatin was only evident as an inhibition of the restoration of sensitivity to this toxin.

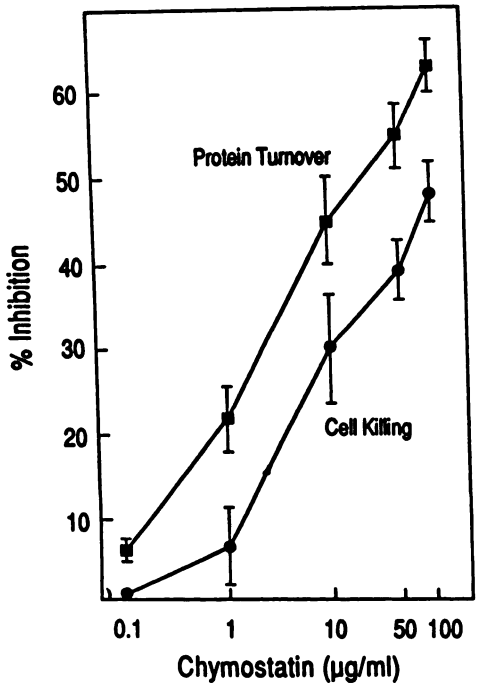

Fig. 3. Dose dependence of the ability of chymostatin to inhibit both protein turnover and the restoration of sensitivity to TBHP. Hepatocytes that had been pretreated with deferoxamine were placed in an insulin-free Krebs-Ringer bicarbonate buffer with or without chymostatin. After 2.5 hr, the cells were treated with TBHP. Other cultures were labeled with [^{14}C]valine for 24 hr, washed three times, and placed in fresh serum-free Williams' E medium containing 2 mM valine, for 1 hr. The cells were washed again and placed in insulin-free Krebs-Ringer bicarbonate buffer. Protein turnover was measured by the net release of [^{14}C]valine over a 2.5-hr period in the presence or absence of chymostatin. Percentage of inhibition was calculated as the ratio of dead cells or protein turnover in the presence of chymostatin to that in its absence. Complete restoration of sensitivity represents the killing by TBHP of 58% of the hepatocytes. In the absence of chymostatin, the rate of protein turnover (0% inhibition) was 4.7%/hr. The results are the mean ± standard deviation of the determinations on three separate cultures.

Concentrations of chymostatin between 1 and 100 μg/ml had an increasingly greater inhibitory effect on the restoration of sensitivity to TBHP. Of further note is the close parallel between the slopes of the two dose-response curves illustrated in Fig. 3, namely the increasing inhibition of protein turnover and the increasing inhibition of the restoration of sensitivity to TBHP.

3-Methyladenine inhibits restoration of sensitivity. 3-Methyladenine is another inhibitor of the autophagic degradation of protein in rat hepatocytes (29). Formation of the autophagosome is the first step in this pathway. Morphometric analysis of cultured hepatocytes treated with 3-methyladenine revealed a marked reduction in the volume density of autophagosomes (29), a result suggesting that 3-methyladenine inhibits the first step in the autophagic degradation of protein.

Fig. 4 details an experiment similar to that shown in Fig. 3. In this case, the effect of increasing concentrations of 3-methyladenine on both protein degradation and the restoration of

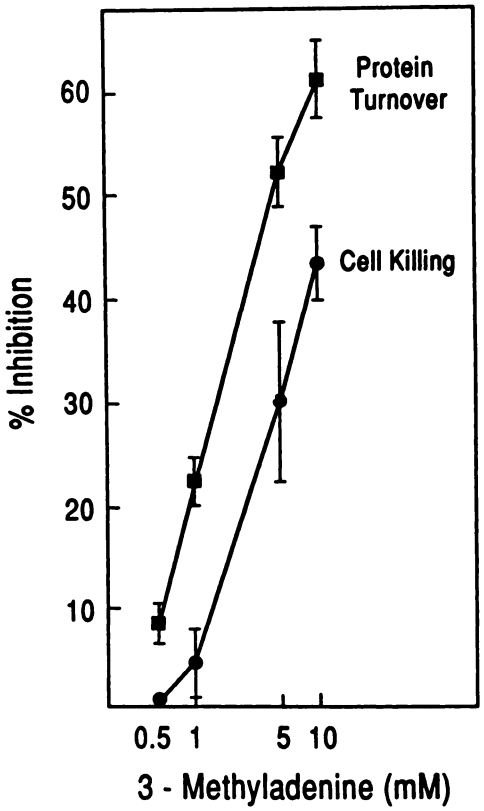

Fig. 4. Dose dependence of the ability of 3-methyladenine to inhibit both protein turnover and the restoration of sensitivity to TBHP. The results are the mean ± standard deviation of the determinations on three separate cultures.

sensitivity to TBHP was evaluated. Interestingly, the slopes of the two dose-response curves in Fig. 4 were again parallel and different from those in response to chymostatin (Fig. 3). 3-Methyladenine had no effect on the toxicity of TBHP in the absence of the pretreatment with deferoxamine.

Benzyl alcohol and colchicine inhibit restoration of sensitivity. Benzyl alcohol is a membrane-perturbing agent that can inhibit the autophagic degradation of proteins by also preventing the formation of autophagic vacuoles (30, 31). Cultured hepatocytes treated with benzyl alcohol showed a striking reduction in the number and volume density of autophagosomes as well as secondary lysosomes (30, 31), a result that paralleled the reduction in the rate of degradation of protein (30, 31). Fig. 5 shows that increasing concentrations of benzyl alcohol inhibited the restoration of sensitivity to TBHP of hepatocytes that were pretreated with deferoxamine. Benzyl alcohol had no effect on the toxicity of TBHP in the absence of the pretreatment with deferoxamine.

The autophagic degradation of protein is dependent on intact microtubules. Accordingly, inhibitors of microtubular function, such as colchicine and vinblastine, inhibit the degradation of liver proteins (32). Colchicine similarly prevented the restoration of sensitivity to TBHP in hepatocytes that had been pretreated with deferoxamine (Fig. 6). In addition, the dose dependency of the inhibition of restoration closely paralleled the inhibition of protein turnover. Colchicine alone had no effect on the toxicity of TBHP in the absence of deferoxamine. Five millimolar colchicine interfered with the measurement of lactate dehydrogenase, and its effect on the restoration of sensitivity could not be evaluated. Vinblastine has significant antioxidant activity and protects cultured hepatocytes from the toxicity of TBHP (data not shown). Thus, its ability to prevent

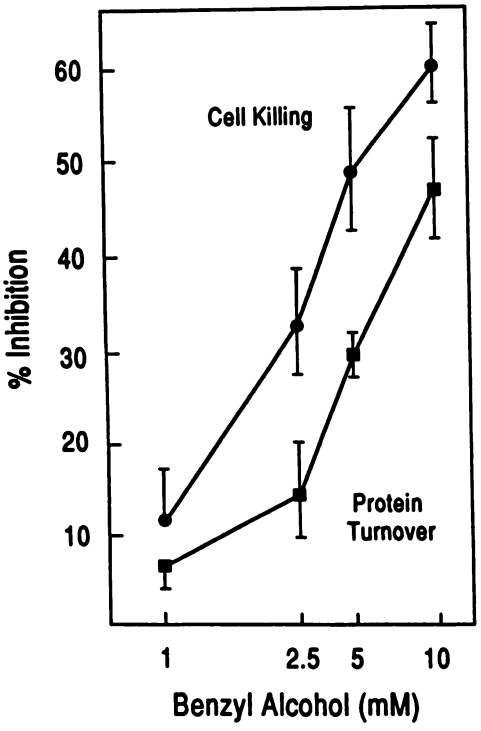

Fig. 5. Dose dependence of the ability of benzyl alcohol to inhibit both protein turnover and the restoration of sensitivity to TBHP. The results are the mean ± standard deviation of the determinations on three separate cultures.

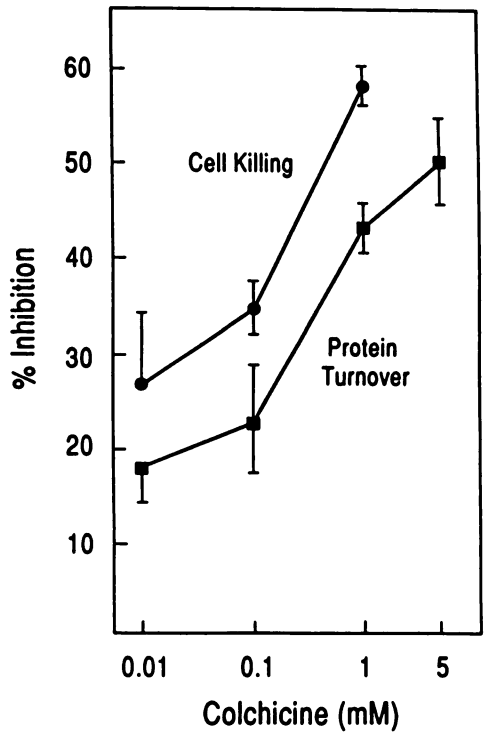

Fig. 6. Dose dependence of the ability of colchicine to inhibit both protein turnover and the restoration of sensitivity to TBHP. The results are the mean ± standard deviation of the determinations on three separate cultures.

TABLE 1

Inhibition of protein degradation and the restoration of cell killing by TBHP

Treatment	Inhibition	
	Protein degradation	Cell killing
	%	
Krebs Ringer	0	0
Williams' E	30 ± 4	38 ± 5
Chymostatin (100 μg/ml)	62 ± 3	47 ± 5
3-Methyladenine (10 mM)	61 ± 4	46 ± 7
Benzyl alcohol (10 mM)	46 ± 3	59 ± 4
Colchicine (1 mM)	42 ± 2	58 ± 2
Oligomycin (10 ng/ml)	41 ± 3	57 ± 5
Methylamine (10 mM)	63 ± 2	52 ± 5
Ascorbic acid (40 μM)	0	41 ± 5

the restoration of sensitivity to this toxin in cells that were pretreated with deferoxamine could not be evaluated.

Oligomycin and methylamine inhibit restoration of sensitivity to TBHP. The maximum inhibition of both protein degradation and the restoration of sensitivity to TBHP by the conditions studied above is summarized in Table 1. In addition, Table 1 documents the effect of two additional inhibitors of the autophagic degradation of protein on the restoration of sensitivity to TBHP. The autophagic degradation of protein is an energy-dependent process (33). Accordingly, oligomycin (Table 1) inhibited both the degradation of protein and the restoration of sensitivity. Finally, lysosomotropic amines, such as methylamine, inhibit protein degradation in rat hepatocytes by alkalinizing acidic vacuolar compartments (11). Methylamine inhibited the restoration of sensitivity to TBHP (Table 1) and inhibited the degradation of protein.

Effect of ascorbic acid on the restoration of sensitivity to TBHP. The autophagic degradation of protein presumably relates to the restoration of sensitivity to TBHP as a result of the release of iron from the turnover of proteins related to the intracellular metabolism of iron. Ferritin is the most abundant iron storage protein in hepatocytes. Thus, the autophagic degradation of ferritin is a likely basis for the formation of the pool of ferric iron required for the cell killing by TBHP. Interestingly, the autophagocytosis of ferritin is inhibited by ascorbic acid without a general effect on proteolysis (34, 35).

Incubation of deferoxamine-pretreated hepatocytes with 40 μM ascorbic acid inhibited the restoration of sensitivity to TBHP by 41% (Table 1). In contrast to all of the other agents that similarly prevented the restoration of sensitivity, this effect of ascorbic acid was not accompanied by an inhibition of the general degradation of prelabeled protein (Table 1). Ascorbic acid (40 μM) did not prevent the killing by TBHP of hepatocytes that were not pretreated with deferoxamine.

Ferritin turnover in cultured hepatocytes. The rate of degradation of ferritin in the cultured rat hepatocytes was determined under the conditions used for the present studies. The cells were labeled with 1 μCi/ml [^{59}Fe]ferric chloride during the initial 24 hr in culture. The cells were then washed, treated with deferoxamine, and divided into two groups. The first group was immediately used to determine the content of radiolabeled ferritin that could be immunoprecipitated from whole-cell sonicates. The second group was cultured for 2.5 hr in Krebs-Ringer bicarbonate buffer, after which time radiolabeled ferritin was immunoprecipitated from similar sonicates. The 2.5-hr incubation reduced by 6% the immunoprecipitable radioactivity (data not shown). Such a decrease corresponds to a half-life of about 22 hr in Krebs-Ringer buffer or 1.3 days in Williams' E medium, a time consistent with the rate of the turnover of rat liver apoferritin reported previously (36, 37). However, the maximum inhibition of 50 to 60% of the restoration of cell killing by TBHP by the agents in Table 1 did not allow an effect on the degradation of [^{59}Fe]ferritin to be unequivocally demonstrated in the 2.5-hr time period during which the cells acquired complete sensitivity.

Discussion

The killing of cultured hepatocytes by an acute oxidative stress depends on a cellular source of ferric iron (1). Hepatocytes that were pretreated with the ferric iron chelator deferoxamine, then washed, and placed in a deferoxamine-free culture medium were insensitive to TBHP. This protection afforded by deferoxamine is achieved without any change in the metabolism of TBHP by glutathione peroxidase or in the alteration in intracellular calcium homeostasis that follows the depletion of glutathione (24). We conclude that the pretreatment with deferoxamine modifies the hepatocytes by depleting them of a pool of ferric iron required for the killing of the cells by TBHP.

Hepatocytes that were modified by pretreatment with deferoxamine reacquired sensitivity to TBHP by their simple incubation in a culture medium containing as little as 0.25 nM ferric iron (Fig. 1). Importantly, this restoration of sensitivity is the result of a process the consequence of which could be reversed by a second treatment with deferoxamine. Thus, the restoration of sensitivity seemingly represents the regeneration of an intracellular pool of deferoxamine-chlelatable ferric iron that is required for the cell killing by TBHP.

The regeneration of this requisite pool of iron occurs in the presence of minimal extracellular iron, namely 0.25 nM $FeCl_3$. Thus, either 0.25 nM $FeCl_3$ is a sufficient extracellular concentration of iron, or the regeneration process is independent of the iron content of the culture medium. The mechanisms of action of the conditions and agents that accelerated or inhibited the regeneration of the intracellular iron pool suggest that this process is entirely a consequence of the mobilization of intracellular iron stores.

Restoration of the sensitivity of deferoxamine-pretreated hepatocytes to TBHP could be inhibited by the addition to the culture medium of a number of compounds, including chymostatin, 3-methyladenine, benzyl alcohol, colchicine, and oligomycin. These compounds differ widely in their chemical composition and may have a variety of effects on cells. However, they all shared two properties. Firstly, none of the agents studied had any protective effect against the toxicity of TBHP for native non-deferoxamine-pretreated hepatocytes. Secondly, they all inhibited the autophagic degradation of protein. Furthermore, with each of the agents studied, the dose-response relationship between the inhibition of protein degradation and the inhibition of the restoration of sensitivity to TBHP was closely parallel. Whereas the slope of the dose-response relationship for the inhibition of protein degradation varied with the particular agent, this slope closely paralleled that of the inhibition of the restoration of sensitivity in each case examined. Thus, it would seem unlikely that the common effect of the six agents studied was related to an action other than the inhibition of protein degradation. We conclude that the regeneration of the pool of ferric iron required for the cell killing with TBHP occurs by the autophagic degradation of a protein or proteins.

This conclusion is supported by the observation that removal of amino acids from the culture medium accelerated the rate of restoration of sensitivity to TBHP of hepatocytes pretreated with deferoxamine. Amino acid starvation activates protein degradation, a result shown above to accompany the enhanced rate of restoration of sensitivity.

Ferritin is a likely candidate for the particular protein whose autophagic degradation regenerates the pool of ferric iron required for the toxicity of TBHP. Ferritin is the major intracellular storage form of iron. That the iron content of ferritin can be readily mobilized is indicated by the absence of storage iron with iron deficiency and the reduction of excess iron stores by phlebotomy in hemochromatosis. However, the cellular mechanisms mediating the release of iron from ferritin are still obscure.

Two mechanisms have been proposed for the mobilization of ferritin iron. It is widely held that the release of iron from intact ferritin can occur by a process that involves reduction of ferric to ferrous iron without a requirement for the degradation of the apoprotein. Under *in vitro* conditions, many potentially physiological or clearly nonphysiological reducing agents are capable of releasing iron from ferritin. However, definitive identification of the reductant(s) that operates within the intact cell has not been accomplished.

Interestingly, the suggestion has been made that an oxidative stress itself may reductively release iron from ferritin, and thereby, promote cell injury. Superoxide anions generated by

xanthine oxidase or by stimulated polymorphonuclear leukocytes can reductively release iron from ferritin (38, 39). Furthermore, the redox cycling of paraquat was demonstrated to promote iron release from ferritin by oxygen-dependent and oxygen-independent mechanisms (40). Thus, the generation of both superoxide anions (oxygen-dependent) and the paraquat free radical itself (oxygen-independent) mediates iron release from ferritin, a process that may contribute to the toxicity of such chemicals.

However, it is unlikely that a reductive release of iron from ferritin by superoxide anions can account for the restoration of sensitivity to TBHP studied here. The ability of the manipulations to inhibit or enhance this process as shown cannot be explained either by an effect on the constitutive rate of generation of superoxide anions or by their ability to release iron from ferritin.

Iron is also liberated from ferritin as a consequence of the intracellular degradation of this protein. The turnover of ferritin involves the organelles of the autophagic vacuolar apparatus (41). Intracellular ferritin is engulfed within autophagic vacuoles by the folding of the endoplasmic reticulum around portions of the cell sap. These vacuoles then fuse with lysosomes to become autophagosomes, organelles within which the ferritin is degraded. The relatively rapid turnover of ferritin protein in the liver is similar to that of other liver proteins (42). The half-life of the apoprotein of rat liver ferritin has been reported to be between 1 and 2 days (36, 37). This agrees with the estimated turnover of ^{59}Fe-labeled ferritin in cultured rat hepatocytes measured in the present report. This would suggest that the rates of turnover of the iron and apoprotein components of ferritin are similar and likely occur by the same mechanism.

The conditions that were used to manipulate the regeneration of the iron pool required to restore sensitivity of the hepatocytes to TBHP generally affected the autophagic degradation of protein. Ascorbate was the only agent that prevented this restoration without a parallel effect on the degradation of total protein. The dose of ascorbate that inhibited restoration of sensitivity to TBHP had no protective effect on the toxicity of this agent for cells that had not been pretreated with deferoxamine. Thus, the effect of ascorbate cannot be attributed to its known action as an antioxidant. Interestingly, ascorbate can block the degradation of cytoplasmic ferritin by reducing the lysosomal autophagy of this protein (34, 35). This action of ascorbate was not accompanied by a general inhibition of proteolysis (35).

It requires emphasis that the data implicating the autophagic degradation of ferritin in the generation of the iron pool do not exclude the existence of other mechanisms. In particular, it will be of interest to attempt to manipulate those mechanisms that may mobilize iron independently of the degradation of ferritin. Investigating whether physiologic or nonphysiologic reducing agents can accelerate the restoration of sensitivity of deferoxamine-treated hepatocytes to TBHP may be a useful approach to this question.

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Send reprint requests to: John L. Farber, Department of Pathology, Room 203 A, Main Building, Thomas Jefferson University, Philadelphia, PA 19107.