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Effects of Vitamin E on the Killing of Cultured Hepatocytes by *tert*-Butyl Hydroperoxide

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SUMMARY

The disposition of vitamin E was examined in cultured rat hepatocytes intoxicated with *tert*-butyl hydroperoxide (TBHP). Culturing of the cells overnight (18–20 hr) with approximately 60 nM α-tocopherol (α-T) equivalents [Williams' E medium, 18 nM tocopherol phosphate (α-TP), 9% fetal calf serum, 43 nM α-T] resulted in a content of α-T that was 16% of the concentration of vitamin E measured in freshly isolated hepatocytes. Supplementation of the medium with 1 μM α-TP maintained the α-T concentration of the cultured cells at the level of freshly isolated hepatocytes. Supplemented hepatocytes exposed to TBHP showed decreased lipid peroxidation and delayed cell killing, compared with hepatocytes not cultured overnight with α-TP. Killing of the supplemented cells by TBHP was accompanied by a loss of α-T. Pretreatment of supplemented hepatocytes with the iron chelator deferoxamine prevented much of the loss of α-T. At the same time, deferoxamine inhibited both the lipid peroxidation and cell killing. The antioxidant *N,N'*-diphenyl-1,4-phenylenediamine reduced the loss of α-T and significantly decreased lipid peroxidation. In the presence of *N,N'*-diphenyl-1,4-phenylenediamine, cell killing was delayed by 15 min and reduced in extent. Overnight supplementation of hepatocytes with nonesterified α-T, or vitamin E esters other than α-TP, similarly rendered the cells less sensitive to TBHP. The nonesterified α-T produced a higher cell-associated vitamin E concentration than did the esters; however, nonesterified α-T did not result in greater protection against TBHP. These data indicate that the mechanisms of the cell killing by TBHP are the same in cultured hepatocytes that contain low or physiological concentrations of vitamin E.

Vitamin E is a family of eight lipophilic compounds, which are obtained from the diet through the ingestion of fats and oils (1, 2). Each tocopherol contains a chromanol head and a C_{16} phytyl side chain. The lipophilic phytyl side chain anchors the molecule in the membrane (3), whereas the chromanol head group, the reactive portion of the molecule, is oriented near the membrane surface (4, 5).

α-T, the most common of the tocopherols in mammals, is thought to be the major antioxidant found in membranes (1, 4, 6). α-T inhibits the propagation of lipid peroxidation by donating a phenolic hydrogen from the 6-position on the chromanol head group to an oxygen- or carbon-centered acyl radical (1, 2, 5, 6). α-T may be viewed as the last line of defense against oxidative cell injury, after other systems, such as catalase, superoxide dismutase, and glutathione peroxidase, are overwhelmed.

Lipid peroxidation is one of the best known manifestations of oxidative cell injury. Nevertheless, the role that lipid peroxidation plays in the pathogenesis of irreversible cell injury with an acute oxidative stress has been a matter of continued debate (7–10). In particular, there has been a tendency to dismiss lipid peroxidation as a significant factor in the pathogenesis of lethal cell injury by activated oxygen species (9, 11, 12). However, in one particular model of oxidative cell injury, namely, the iron-dependent killing of cultured hepatocytes by TBHP, recent evidence has shown that the peroxidation of membrane lipids does, in fact, mediate the death of the cells (13).

Thus, it was of interest to examine the metabolism of α-T in cultured hepatocytes intoxicated with TBHP. Cultured cells have been used to study the uptake, intracellular distribution, and secretion of vitamin E (14–16). Several reports have considered the effects of oxidative injury on vitamin E metabolism in suspensions of freshly isolated hepatocytes (17–23). However, limitations exist for such systems as models of oxidative injury (24, 25). The present study represents the first attempt to detail the role of α-T in cultured hepatocytes under conditions where lipid peroxidation has been causally linked to the death of the cells.

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ABBREVIATIONS: α-T, α-tocopherol; α-TA, α-tocopherol acetate; α-TP, α-tocopherol phosphate; α-TS, α-tocopherol succinate; ANOVA, analysis of variance; Me_2SO, dimethyl sulfoxide; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); LDH, lactate dehydrogenase; SNK, Student-Neuman-Keuls *post hoc* test; TBHP, *tert*-butyl hydroperoxide; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.

We report below that cultured hepatocytes must be supplemented overnight with vitamin E in order to maintain physiological concentrations of α-T. With such supplementation, we have examined the relationship between the cellular content of α-T and the time course of the manifestations of iron-dependent oxidative cell injury.

Materials and Methods

Hepatocytes. Male Sprague-Dawley rats (150–200 g) obtained from Charles River (Wilmington, MA) were housed for at least 1 week in quarters approved by the American Association of Laboratory Animals, allowed food (Purina Rodent Laboratory Chow 5001) and water *ad libitum*, and fasted overnight before use. Isolated hepatocytes were prepared by collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) perfusion, as described by Seglen (26). Yields of 2–4 × 10^8 cells/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 Glass Works, Corning, NY), at a density of 1.33 × 10^6 cells/flask, in 3 ml of Williams' E medium (GIBCO Laboratories, Grand Island, NY) containing 9.1 IU/ml penicillin, 9.1 μg/ml streptomycin, 47 μg/ml gentamicin sulfate, 0.018 unit/ml insulin, and 9% heat-inactivated (55° for 15 min) fetal calf serum (JRH Biosciences, Kansas City, MO) (complete Williams' E medium).

After a 2-hr incubation at 37° in an atmosphere of 5% CO_2/95% room air, the cultures were rinsed twice with 3 ml of prewarmed HEPES buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10.1 mM HEPES, pH 7.4), to remove any unattached or dead cells. Fresh complete Williams' E medium (5 ml), with or without a final concentration of 1 μM α-TP, disodium (Sigma Chemical Co., St. Louis, MO), was added, and the hepatocytes were incubated overnight.

Overnight cultures (18–20 hr) were washed twice with 3 ml of warm HEPES buffer. Five milliliters of Williams' E medium without fetal calf serum (incomplete Williams' E medium) were added to cultures. The 1 μM α-TP that was present in the overnight cultures was omitted from the medium during all experiments. TBHP (Sigma) was diluted in medium and added to the cultures (1% volume). Cells were incubated under 5% CO_2/95% air, at 37°. The concentrations of TBHP and times of sampling are indicated in the figure legends.

In a separate series of experiments, overnight cultures supplemented with 1 μM α-TP were pretreated with a final concentration of 12 mM deferoxamine mesylate (CIBA Pharmaceutical Co., Summit, NJ) for 1 hr before washing with HEPES buffer. Deferoxamine was dissolved in deionized water and added to the cultures in a volume of 4%. After the cells were washed twice with 3 ml of prewarmed HEPES buffer, 5 ml of incomplete Williams' E medium were added to the flasks, and the cells were treated with 750 μM TBHP for 90 min.

In another series of experiments, the hepatocytes were cultured overnight with 1 μM α-TP and then incubated the next day in the presence or absence of a final concentration of 1 μM DPPD (Aldrich Chemical Co., Milwaukee, WI). Cells in culture for 18–20 hr were washed twice with 3 ml of warm HEPES buffer, and 5 ml of incomplete Williams' E medium were added. DPPD was dissolved and diluted in Me_2SO (Sigma) and was added to the cultures in a volume of 0.5%. Me_2SO in this volume has no effect on cultured cells. Cultures were treated with 750 μM TBHP for 90 min.

A final series of cultures examined the effects of TBHP on hepatocytes supplemented with nonesterified α-T or one of several tocopherol esters. Hepatocytes were cultured overnight with 1 μM concentrations of one of the following: α-T, α-TP, α-TS, or α-TA (Sigma). The compounds were dissolved and diluted in Me_2SO, with the exception of α-TP, which was dissolved in water and diluted in Me_2SO. The compounds were added to flasks in a volume of 0.5%. After 18–20 hr, cultures were washed twice with warmed HEPES buffer, and 5 ml of incomplete Williams' E medium without additional tocopherol were added. Cells were treated with 500 μM TBHP for 1 hr.

Tocopherol determinations. α-, γ-, and δ-Tocopherol (Sigma) were determined by modification of the methods of Burton *et al.* (27) and Liebler *et al.* (28). Briefly, the flasks were washed twice with 3 ml of prewarmed HEPES buffer. Two milliliters of 50% ethanol containing 0.1 mg/ml butylated hydroxytoluene were added to the flasks, and the cells were scraped from the plates. The cells were sonicated on ice for 15–20 sec, and 250 pmol of the internal standard, δ-tocopherol (90%), were added. The sonicated cells were extracted with 2 ml of hexane, vortexed for 1 min, and centrifuged for 2 min. The top layer of hexane was removed, and the extraction procedure was repeated a second time. The hexane was evaporated to dryness under nitrogen, and the residue was dissolved in 500 μl of methanol. Hexane and methanol were of the highest grade (Optima brand; Fisher Scientific Co., Pittsburgh, PA).

The tocopherols were determined by injection of 100 μl of the methanol resuspension onto a high performance liquid chromatography system (Perkin-Elmer LC 410, with an ISS 100 autoinjector), with a fluorometric detector (Perkin-Elmer LC 240; excitation, 205 nm; emission, 325 nm; fixed factor, 10; response, 4). The conditions for analysis consisted of a mobile phase of 96% methanol/4% water, a flow rate of 2 ml/min, and a Supelcosil LC-18 column (150 mm × 4.6 mm; 3-μm packing), with a C_{18} precolumn (Supelco Inc., Bellefonte, PA). Quantitation of α- and γ-tocopherol were determined with a Nelson series 900 interface with model 2100 PC integrator (version 5.1), by using the area ratio of the unknown tocopherol to δ-tocopherol. The standard curve was based on the area ratios of known concentrations of tocopherols to δ-tocopherol. The standard curve for γ-tocopherol was corrected for any γ-tocopherol contributed by the addition of internal standard; correction for α-T was not necessary. δ, γ, and α-tocopherol had retention times of 5.2, 6.2, and 8.1 min, respectively.

Other assays. Cell killing was determined by release of LDH into the medium. The change in absorbance of NADH at 340 nm was measured for 30 sec on a Perkin-Elmer Lambda 1 UV/visible spectrophotometer. One hundred percent release of LDH was determined after lysis of hepatocytes in a set of control cultures with Triton X-100 (0.5% final concentration) (Sigma). The LDH reaction mixture contained 2.7 ml of 100 mM potassium phosphate buffer (pH 7.5) with 85 μM NADH, 100 μl of 23 mM sodium pyruvate, and 200 μl of sample (29).

Lipid peroxidation, as determined by the accumulation of TBA-reactive products released into the medium, was measured by a modification of the method of Ohkawa *et al.* (30). Briefly, TCA was added to flasks (4.5% final concentration), and the cells were removed by scraping. The scraped cells were sonicated for 10–15 sec and centrifuged to pellet the protein. One milliliter of TCA supernatant (without butanol extraction) was added to 2 ml of TBA solution (composed of 0.45%, w/v, TBA and 7.5%, v/v, acetic acid, pH to 4.15 with 10 N sodium hydroxide), to yield the final concentrations and pH described by Ohkawa *et al.* (30). Samples were placed in a boiling water bath for 15 min, cooled to room temperature, and read fluorometrically on a Perkin-Elmer LS-5 fluorometric spectrophotometer (excitation, 515 nm; emission, 553 nm; slit widths, 5 nm; fixed factor, 60; response, 4). TBA-reactive products were quantified using 1,1,3,3-tetramethoxypropane (Sigma), in TCA-acidified medium, as the standard. Because TBHP and Williams' E medium produced a slight fluorescence after boiling with the TBA solution, a blank sample with TBHP and Williams' E medium was processed, and its value was subtracted from appropriate readings. We found no differences in the final amounts of TBA-reactive products when the TCA supernatants were compared with the butanol extract.

Protein was determined on each sample by the method of Lowry *et al.* (31), using bovine serum albumin as the standard.

Statistical analysis. Data from duplicate or triplicate flasks at each data point were averaged, to obtain a single value for each point in each experiment. The number of times the experiment was repeated (n) is indicated in the figure legends. Data from flasks within a given experiment were considered paired, because all the hepatocytes for one experiment came from a single rat. One- and two-way ANOVA using a repeated measures design, *post hoc* tests (SNK and paired t test), and

Pearson correlation coefficients were performed on data by using the PC version of the SAS statistical package (version 6.04) (32). When unequal variance was encountered, the logarithmically transformed data were analyzed (33). For comparisons between treatments, control values were deleted from the analysis, because they produced significant interactions. Data represent mean ± standard deviation.

Results

Vitamin E content of cultured hepatocytes. The α-T concentration of freshly isolated hepatocytes is shown in Table 1. After culturing of the hepatocytes overnight (18–20 hr) in complete Williams' E medium, the α-T content declined to 16% of its initial value (Table 1). An increase in the α-T content of the culture medium accounted for approximately 40% of the vitamin E lost from the cells (data not shown).

Supplementation of the medium with at least 1 μM α-TP produced a concentration of α-T in cultured cells equivalent to that of freshly isolated hepatocytes. The complete Williams' E medium (9% fetal calf serum, final concentration) used in culturing the hepatocytes contained 0.043 μM α-T. Serum from the rats used in this laboratory contained 14.7 μM α-T, which was in agreement with values obtained by others (34).

The concentration of γ-tocopherol in freshly isolated hepatocytes was 10 ± 3 pmol/mg of protein. γ-Tocopherol concentrations declined to 3 pmol/mg of protein after 18–20 hr, regardless of the presence or absence of 1 μM α-TP.

Vitamin E content of hepatocytes treated with TBHP. Hepatocytes that were cultured overnight with or without 1 μM α-TP in the medium were washed, placed in fresh medium without α-TP, and exposed to increasing concentrations of TBHP. When the viability of the hepatocytes was assessed after 1 hr (Fig. 1A), hepatocytes cultured with α-TP were significantly less sensitive to TBHP than those cultured without α-TP. Nonetheless, the percentage of dead cells in both groups increased as the concentration of TBHP increased. In cells cultured with α-TP, concentrations of TBHP of 250 μM and greater resulted in significant cell killing, whereas in cells cultured without α-TP, concentrations of TBHP of 125 μM and greater were sufficient to cause significant cell killing.

The α-T content of the hepatocytes in Fig. 1A is shown in Fig. 1B. When hepatocytes cultured with α-TP were exposed to TBHP, the cellular content of α-T declined, in a concentration-dependent manner. Concentrations of TBHP of 250 μM and greater resulted in α-T concentrations that were significantly lower than control concentrations. This decline in α-T content correlated ($r = -0.84$; $p < 0.0001$) with the increase in the killing of those cells cultured with α-TP. In other words, 71% (r^2) of the variation associated with the decrease in α-T concentrations is statistically related to the increase in cell killing.

Hepatocytes not cultured overnight with α-TP had α-T concentrations that were 18% of the initial α-T concentration in supplemented cells (Fig. 1B). These hepatocytes showed no change in α-T concentrations after exposure to TBHP.

Time course of oxidative injury in cultured hepatocytes treated with TBHP. The time course of the effects of a toxic concentration of TBHP on three parameters related to oxidative cell injury is illustrated in Fig. 2. Hepatocytes cultured overnight in the presence or absence of 1 μM α-TP were washed, placed in fresh medium without α-TP, and treated with 750 μM TBHP. At the times indicated in Fig. 2, the cellular content of α-T (Fig. 2A), the accumulation of products of the peroxidation of cellular lipids (Fig. 2B), and the loss of viability of the hepatocytes (Fig. 2C) were measured.

The α-T concentrations in hepatocytes cultured with α-TP (Fig. 2A) declined in the first 15 min after exposure to TBHP. By 30 min, the α-T concentration was 55% of its initial value. There was no further decrease in tocopherol concentrations between 30 and 90 min. Hepatocytes cultured without α-TP contained 18% of the initial α-T concentrations and showed no significant change in the α-T concentration over the entire 90-min course of the experiment.

The accumulation of malondialdehyde, as measured by TBA-reactive products in the medium, is a sensitive index of the peroxidation of cellular lipids in cultured hepatocytes intoxicated with TBHP. Lipid peroxidation in hepatocytes cultured overnight with 1 μM α-TP (Fig. 2B) was significantly lower than in hepatocytes cultured without α-TP. However, cells cultured with and without α-TP exhibited significant accumulation of TBA-reactive products within 5 min of the addition of TBHP. Lipid peroxidation in both groups increased in a time-dependent manner.

Data from 15, 30, 45, and 60 min after exposure to TBHP (Fig. 2C) indicated that hepatocytes cultured with α-TP exhibited significantly less cell killing than hepatocytes cultured without α-TP. However, by 90 min both groups had the same percentage of dead cells. Both groups showed significant cell killing at times of 30 min and longer.

Iron dependence of toxicity of TBHP. The killing of cultured hepatocytes by TBHP depends on a source of ferric iron (13, 35, 36). Chelation of the requisite iron pool with deferoxamine prevents the cell killing by TBHP (13, 35, 36). Fig. 3 illustrates the effect of deferoxamine on the same three parameters of oxidative cell injury assessed in Fig. 2. In the experiment illustrated in Fig. 3, all hepatocytes were cultured overnight in the presence of 1 μM α-TP. Deferoxamine was added 1 hr before the cells were washed. The hepatocytes were then placed in fresh medium lacking α-TP and deferoxamine. At the times indicated, the cellular content of α-T (Fig. 3A), the accumulation of products of the peroxidation of cellular lipids (Fig. 3B), and the loss of viability of the hepatocytes (Fig. 3C) were measured.

TABLE 1
α-T concentrations

	α-T	n
	pmol/mg of protein	
Freshly isolated hepatocytes	170 ± 49	24
Cultured hepatocytes (18–20 hr)[a]	27 ± 16[b]	18
Cultured hepatocytes + 1 μM α-TP (18–20 hr)[a]	193 ± 43[c]	21
	α-T	**n**
	nM	
Complete Williams' E medium	43 ± 15	21
Rat serum[d]	14,676 ± 2,024	12

[a] Cultured hepatocytes were incubated overnight in 5 ml of complete Williams' E medium in 95% air/5% CO_2 at 37°. Williams' E medium contains 18 nM α-TP, disodium; complete Williams' E medium has 9.1 IU/ml penicillin, 9.1 μg/ml streptomycin, 47 μg/ml gentamicin, 0.018 unit/ml insulin, and 9% (final concentration) heat-inactivated (55° for 15 min) fetal calf serum.

[b] Significantly less than cultured hepatocytes plus 1 μM α-TP ($p < 0.0001$), as determined by paired t test ($n = 18$).

[c] Not significantly different from freshly isolated hepatocytes, as determined by paired t test ($n = 21$).

[d] Male Sprague-Dawley rats, from Charles River, weighed 328 ± 54 g.

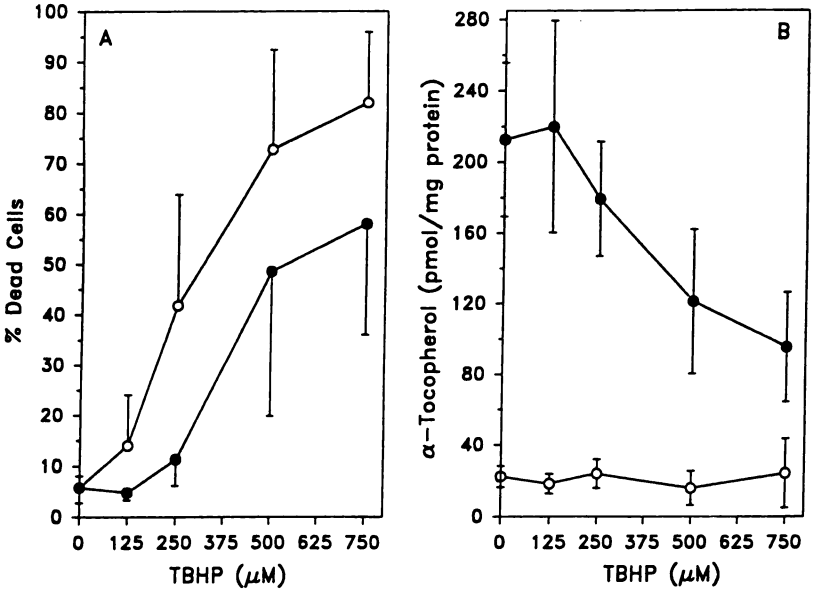

Fig. 1. Effect of TBHP concentration on cell killing (A) and α-T concentration (B) of hepatocytes cultured overnight with (●) and without (○) 1 μM α-TP. Hepatocytes were prepared and incubated overnight with or without 1 μM α-TP. After 18–20 hr, cells were washed, placed in Williams' E medium without serum and without 1 μM α-TP, and then incubated with the designated concentration of TBHP for 60 min. Cells supplemented with α-TP and not treated with TBHP showed no decline in the α-T concentration over the course of 60 min (data not shown). Values are the mean ± standard deviation for the results of five or six experiments. A, Hepatocytes supplemented with α-TP showed less overall killing with TBHP than did cells cultured without α-TP ($p < 0.003$, from two-way ANOVA). In cells cultured with α-TP, concentrations of TBHP of ≥250 μM showed cell killing ($p < 0.01$, compared with control cells, from SNK). Untreated cells showed killing at concentrations of ≥125 μM ($p < 0.001$, compared with control cells, from SNK). B, In cells treated with α-TP, concentrations of TBHP of ≥250 μM resulted in lower α-T concentrations ($p < 0.05$, compared with control cells, from SNK).

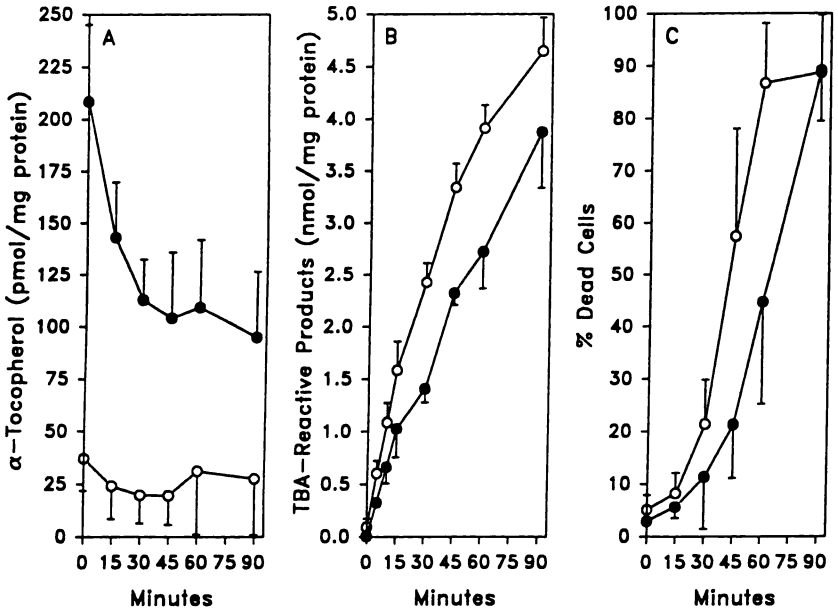

Fig. 2. Effect of 750 μM TBHP on the α-T concentration (A), lipid peroxidation (B), and cell killing (C) of hepatocytes cultured overnight with (●) and without (○) 1 μM α-TP. Hepatocytes were prepared and incubated overnight with or without 1 μM α-TP. After 18–20 hr, cells were washed, placed in Williams' E medium without serum and without 1 μM α-TP, and then incubated with 750 μM TBHP for 90 min. Cells supplemented with α-TP and not treated with TBHP showed no decline in the α-T concentration over the course of 90 min (data not shown). Values are the mean ± standard deviation for the results of three or four experiments. A, After 15 min, cells cultured overnight with α-TP showed a decline within the first 15 min after exposure to TBHP ($p < 0.0001$, compared with zero time, from SNK). B, Cells incubated overnight with α-TP showed an overall lower lipid peroxidation ($p < 0.02$, from two-way ANOVA). By 5 min, both groups showed accumulation of TBA-reactive products greater than zero time (p at least 0.005, from SNK). C, From 15–60 min, hepatocytes treated with α-TP showed an overall lower percentage of cell killing ($p < 0.03$, from two-way ANOVA). In both groups, times of ≥30 min showed greater cell killing than time zero (p at least 0.005, from SNK).

The loss of α-T (Fig. 3A) was significantly less in the hepatocytes pretreated with deferoxamine. Within 15 min, the α-T content of hepatocytes pretreated with deferoxamine declined by 20%, whereas in hepatocytes not pretreated with deferoxamine the α-T content declined by 33%. By 30 min, the α-T concentration in cells pretreated with deferoxamine was still 80% of its initial value; however, the α-T content of cells not pretreated with deferoxamine had decreased further, to 50% of its initial value. There was no significant change in the tocopherol concentration of either group after 30 min.

Pretreatment with deferoxamine (Fig. 3B) prevented the accumulation of TBA-reactive products during the 90-min time course experiment. In contrast, hepatocytes not pretreated with deferoxamine exhibited a time-dependent increase in lipid peroxidation, with a significant accumulation of TBA-reactive products within 5 min after the addition of TBHP.

Pretreatment with deferoxamine (Fig. 3C) also prevented the cell killing by 750 μM TBHP for at least 90 min. In contrast, within 30 min hepatocytes not pretreated with deferoxamine showed significant cell killing, which increased in a time-dependent manner over the next hour.

Lipid peroxidation and the toxicity of TBHP. Two mechanisms have been used to explain the killing of hepatocytes by TBHP. One mechanism is related to the peroxidation of cellular membranes (13). Thus, antioxidants, such as DPPD, prevented the cell death produced by TBHP that was dependent on lipid peroxidation. The second mechanism of cell killing is unaffected by antioxidants, occurs in the absence of lipid peroxidation, and is associated with the loss of mitochondrial function (35).

Fig. 4 illustrates the effect of the antioxidant DPPD on the time course of oxidative cell injury when the α-T content of cultured hepatocytes was maintained at physiological concentrations. Hepatocytes were cultured overnight with 1 μM α-TP, washed, and placed in fresh medium not supplemented with α-TP. Cells were treated with 750 μM TBHP in the presence or absence of 1 μM DPPD.

DPPD (Fig. 4A) significantly reduced the loss of α-T. Within

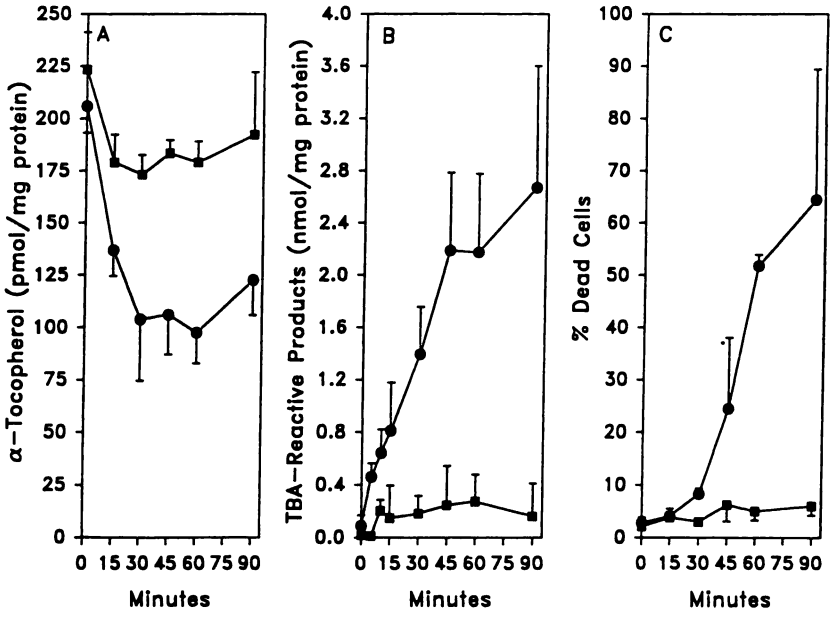

Fig. 3. Effect of 750 μM TBHP on the α-T concentration (A), lipid peroxidation (B), and cell killing (C) of hepatocytes pretreated with (■) and without (●) 12 mM deferoxamine. Hepatocytes were prepared and incubated overnight with 1 μM α-TP. After 18–20 hr, cells were treated with 12 mM deferoxamine. After 1 hr, cells were washed, placed in Williams' E medium without serum and without 1 μM α-TP, and then incubated with 750 μM TBHP for 90 min. Cells supplemented with α-TP and not treated with TBHP showed no decline in the α-T concentration over the course of 90 min (data not shown). Values are the mean ± standard deviation for the results of three or four experiments. A, Hepatocytes pretreated with deferoxamine showed higher overall α-T concentrations ($p < 0.03$, from two-way ANOVA). By 15 min, both groups had lower α-T concentrations than at time zero (p at least 0.05, from SNK). B, Cells not pretreated with deferoxamine showed higher levels of TBA-reactive products than pretreated cells (p at least 0.02, from paired t tests). Cells not pretreated had higher levels than zero time by 5 min ($p < 0.001$, from SNK). C, At times of ≥30 min, cells not pretreated with deferoxamine showed greater cell killing than pretreated cells (p at least 0.05, from paired t test), as well as its zero time ($p < 0.005$, from SNK).

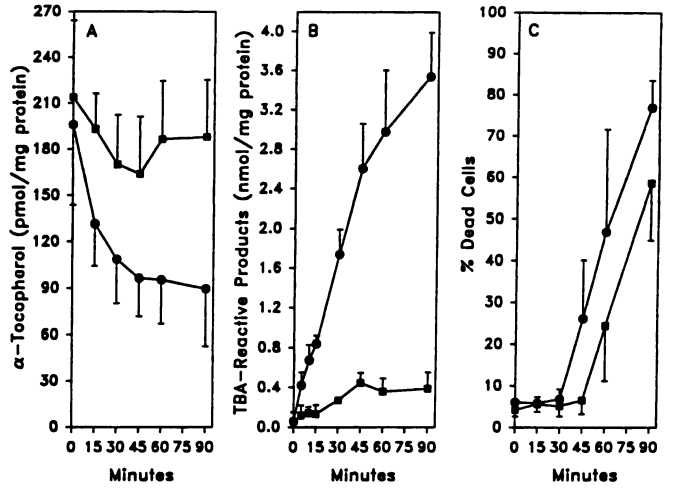

Fig. 4. Effect of 750 μM TBHP on the α-T concentration (A), lipid peroxidation (B), and cell killing (C) of hepatocytes incubated with (■) and without (●) 1 μM DPPD. Hepatocytes were prepared and incubated overnight with 1 μM α-TP. After 18–20 hr, cells were washed and placed in Williams' E medium without serum and without 1 μM α-TP. DPPD (1 μM) and 750 μM TBHP were added for 90 min. Cells supplemented with α-TP and not treated with TBHP showed no decline in the α-T concentration over the course of 90 min (data not shown). Values are the mean ± standard deviation for the results of three to five experiments. A, Hepatocytes treated with DPPD showed higher overall α-T concentrations ($p < 0.002$, from two-way ANOVA). By 15 min, both groups had lower α-T concentration than at time zero (p at least 0.01, from SNK). B, Cells not treated with DPPD showed higher lipid peroxidation than did treated cells by the first 5 min (p at least 0.02, from paired t tests). Compared with zero time, untreated cells showed accumulation of TBA-reactive products by 5 min ($p < 0.0001$, from SNK). There was slight accumulation of TBA-reactive products in the DPPD-treated cells beginning at 30 min ($p < 0.01$, from SNK). C, At times of ≥45 min, cells not treated with DPPD showed greater cell killing than did treated cells ($p < 0.005$, from two-way ANOVA). Cells not treated with DPPD were different from zero time at 45 min ($p < 0.0001$, from SNK), whereas treated cells were not different until 60 min ($p < 0.0001$, from SNK).

15 min, the α-T concentration in cells incubated with DPPD declined by 8%, whereas the α-T concentrations in hepatocytes not incubated with DPPD declined by 32%. By 30 min, the α-T concentration in cells treated with DPPD was 80% of its initial values. However, the α-T concentration in cells not treated with DPPD declined to 56% of its initial values. There was no significant change in the tocopherol content of either group after 30 min.

Treatment with DPPD (Fig. 4B) prevented the accumulation of TBA-reactive products during the first 15 min of the experiment. From 30 to 90 min, there was a small accumulation of TBA-reactive products in DPPD-treated hepatocytes. However, at 90 min this accumulation was 1 order of magnitude lower than in the cultures not treated with DPPD. In the absence of DPPD, lipid peroxidation was evident within 5 min of the addition of TBHP.

At 45 min (Fig. 4C), 26% of the hepatocytes not treated with DPPD had died, whereas no significant cell killing had occurred in hepatocytes treated with DPPD. Between 45 and 90 min, the number of dead cells increased at the same rate in the presence or absence of DPPD. At these times, cells treated with DPPD exhibited lower cell killing than cells not treated with DPPD.

α-T, tocopherol esters, and toxicity with TBHP. Fig. 5 shows the effect of TBHP on hepatocytes cultured overnight with 1 μM nonesterified α-T or 1 μM concentrations of one of the following esters of tocopherol: α-TP, α-TS, or α-TA. After 18–20 hr, hepatocytes were washed, placed in fresh medium without tocopherol supplementation, and treated with 500 μM TBHP for 1 hr. The α-T concentrations and the viability of the cells before and after exposure to TBHP were determined.

Exposure to 500 μM TBHP for 1 hr caused significant cell killing (Fig. 5A), whether or not the cells were cultured with tocopherol or a tocopherol ester. However, overnight supplementation of the medium with α-T or one of its esters reduced the percentage of dead cells to less than half the percentage of dead cells seen in the absence of such supplementation. There was no significant difference between α-T and any tocopherol ester in the protection against 500 μM TBHP.

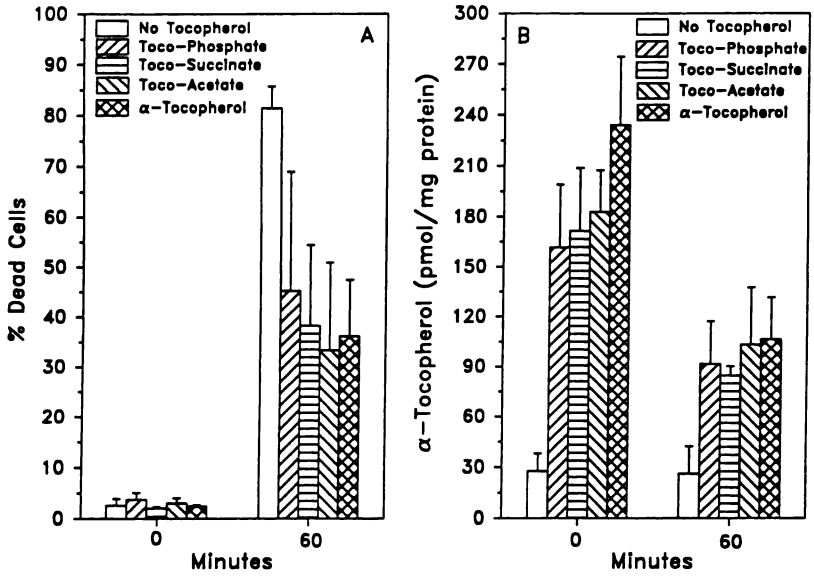

Fig. 5. Effect of 500 μM TBHP on the cell killing (A) and α-T concentration (B) of hepatocytes cultured overnight with 1 μM α-T or one of its esters. Hepatocytes were prepared and incubated overnight without supplementation or with 1 μM α-TP, α-TS, α-TA, or nonesterified α-T. After 18–20 hr, cells were washed, placed in Williams' E medium without serum and without tocopherol equivalents, and then incubated with 500 μM TBHP for 60 min. Values are the mean ± standard deviation for the results of four experiments. A, Although exposure to TBHP caused significant cell killing in all groups ($p < 0.03$, from two-way ANOVA), cells given tocopherol or one of its esters showed less killing, compared with unsupplemented cells ($p < 0.0001$, from SNK). B, Cells supplemented with nonesterified α-T had higher concentrations of α-T than cells supplemented with tocopherol esters ($p < 0.0001$, from SNK). Although exposure to TBHP reduced α-T in all supplemented groups ($p < 0.003$, from two-way ANOVA), these groups were still greater than the unsupplemented group ($p < 0.005$, from SNK).

Hepatocytes supplemented with α-T or one of its esters contained initial α-T concentrations (Fig. 5B) significantly greater than those in hepatocytes that were not supplemented. Cells exposed to 1 μM nonesterified α-T had higher cell-associated concentrations of α-T than did cells given 1 μM tocopherol ester.

Exposure to 500 μM TBHP for 1 hr significantly decreased the α-T contents of the supplemented groups, to an average of 48% of their initial values, but had no effects on the tocopherol content of unsupplemented cells (*t* test). The cellular α-T concentrations did not differ significantly between the supplemented groups. However, the tocopherol concentrations of the supplemented cells were still greater than the tocopherol concentrations of unsupplemented cells.

Discussion

Several conclusions can be drawn from the data presented above. Within 18–24 hr, a depletion of cellular α-T concentrations occurred when hepatocytes were cultured in a widely used tissue culture medium (Williams' E). Supplementation of the medium with at least 1 μM nonesterified α-T or an α-T ester maintained the physiological content of vitamin E in cultured hepatocytes. Like tocopherol-deficient hepatocytes, hepatocytes with normal concentrations of tocopherol were still susceptible to an iron-dependent oxidative injury, although to a lesser extent.

The secretion of α-T seems the likely basis for the depletion of cellular vitamin E reported here. The liver normally secretes α-T as a component of very low density lipoproteins (1, 2, 14, 37, 38). Hepatocytes continue to secrete α-T in culture (14, 15), and their content of vitamin E declines under these conditions (14, 15). As in those studies, the cells in the present study showed a decrease while the medium showed an increase (data not shown) in α-T concentration after 18–20 hr of incubation.

As mentioned, nonesterified α-T protected cultured hepatocytes from an oxidative stress to the same extent as did the tocopherol esters (Fig. 5). This result differs from that obtained with suspensions of freshly isolated hepatocytes (20–22). Isolated hepatocytes incubated with or without nonesterified α-T were not resistant to doxorobicin (20), ethyl methanesulfonate (21), or 95% oxygen toxicity (22). However, incubation with 25 μM α-TS protected isolated cells. Nonesterified α-T (25 μM) did not protect, even though the isolated cells contained concentrations of cell-associated vitamin E that were higher than those obtained with 25 μM α-TS. Thus, an important difference exists between the disposition of α-T in cultured and isolated hepatocytes.

Because α-T, not its esters, is the physiological antioxidant in membranes, the inability of isolated hepatocytes to utilize free α-T represents a significant limitation of the usefulness of these cells. Cultured hepatocytes morphologically resemble actual liver tissue and contain intact membrane functions (25, 39, 40), factors that may be important for the intracellular metabolism of α-T. In addition, the lipoproteins in the calf serum added to Williams' E medium may facilitate a physiological uptake and intracellular localization of vitamin E and its esters by the cultured hepatocytes (1, 2, 18).

The finding that hepatocytes are depleted of vitamin E when cultured in Williams' E medium plus calf serum (Table 1; Fig. 1) is also important with regard to the interpretation of previous work from this laboratory. A number of chemicals readily killed cultured hepatocytes in the presence but not in the absence of extracellular calcium (41–44). In contrast, suspensions of isolated hepatocytes were more sensitive to toxins in the absence than in the presence of extracellular calcium (17, 19, 45–48).

These latter results were obtained in the absence of α-TS in the buffer medium used to suspend the isolated hepatocytes (17, 19, 45–48). In the presence of 25 μM α-TS, isolated hepatocytes were more sensitive to toxins when calcium was included in the medium (17, 19, 21). Also in the presence of 25 μM α-TS, the isolated hepatocytes accumulated more α-T when extracellular calcium was absent (17–21). Thus, in the presence of vitamin E, the protective effect of a calcium-free medium on isolated hepatocytes was attributed to their enrichment with α-T, rather than a direct effect of calcium (18).

Similarly, the decreased cell killing in the absence of extracellular calcium previously reported with cultured hepatocytes (41–44) was argued to depend upon an increase in the vitamin E content of the cells (18). It was assumed that the concentration of α-TP in Williams' E medium was sufficient to enrich

the cultured hepatocytes with α-T. Clearly, the results of the present study do not permit this interpretation. In contrast to what was assumed (18), hepatocytes cultured overnight in Williams' E medium plus 9% calf serum are depleted of α-T. Thus, in earlier reports using cultured hepatocytes (41–44), the cell killing reflects a true dependence on extracellular calcium.

As was demonstrated previously with tocopherol-deficient hepatocytes (13), the inclusion of the antioxidant DPPD in the incubation medium also prevented lipid peroxidation in hepatocytes with physiological α-T concentrations. However, unlike in tocopherol-deficient cells (35), DPPD did not prevent the killing of tocopherol-sufficient cells (Fig. 4). This seeming discrepancy is explained by the greater concentration of TBHP needed to kill the tocopherol-sufficient hepatocytes in the current study. The killing of cells in the presence of DPPD was attributable to mechanisms involving mitochondrial dysfunction rather than lipid peroxidation (35). At a concentration of 250 μM TBHP, tocopherol-deficient cells incubated with DPPD were completely protected against cell killing, whereas cells not incubated with DPPD were not (13). In the present study, tocopherol-sufficient cells treated with 250 μM TBHP and incubated with or without DPPD showed no cell killing (data not shown). The absence of α-T in the former cells (13) made these cells highly susceptible to cell killing by lipid peroxidation. However, 1 μM DPPD was adequate to protect against the lipid peroxidation produced by 250 μM TBHP. In the latter cells (tocopherol sufficient), the physiological concentration of α-T was enough to protect against 250 μM TBHP, and DPPD provided little additional protection. Thus, in tocopherol-sufficient cells, it was necessary to use higher concentrations of TBHP to produce injury.

The 750 μM TBHP used to kill tocopherol-sufficient hepatocytes produces both lipid peroxidation-dependent and -independent cell injury. With higher concentrations of TBHP, cell killing by mechanisms that affect mitochondrial function increases (35). Consequently, the protection against peroxidation provided by physiological concentrations of α-T and other antioxidants, such as DPPD, becomes less effective. The difference between the cell killing curves in Fig. 4C is attributable to peroxide-independent mechanisms, because incubation with DPPD prevented damage due to lipid peroxidation (Fig. 4B).

In conclusion, for study of the effects of α-T on oxidative injury, hepatocytes in culture require supplementation with vitamin E or one of its esters, in order to maintain normal physiological concentrations of cellular α-T. However, cultured hepatocytes lacking physiological concentrations of α-T are a useful model to study the peroxidation-dependent and peroxidation-independent mechanisms of cell injury. The lack of endogenous membrane antioxidant (α-T) in those hepatocytes allows excellent experimental separation of these two known mechanisms of oxidative cell injury.

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