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Independent Antioxidant Action of Vitamins E and C in Cultured Rat Hepatocytes Intoxicated with Allyl Alcohol

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ABSTRACT. The relationship between the metabolism of α-tocopherol (vitamin E) and ascorbate (vitamin C) was examined in cultured hepatocytes intoxicated with allyl alcohol. Alcohol dehydrogenase rapidly metabolizes allyl alcohol to the potent electrophile acrolein. Acrolein depletes the glutathione (GSH) content of the hepatocytes, thereby sensitizing the cells to the constitutive flux of activated oxygen species. Supplementation of the medium with 1 μM α-tocopherol phosphate (α-TP) prevents the 85% decline in cellular vitamin E seen after 16–18 hr in culture. In cells supplemented with α-TP, allyl alcohol produced a concentration-dependent decline in the cellular content of α-tocopherol, and these cells were more resistant to cell killing than hepatocytes not supplemented with α-TP. α-TP concentrations that raised the cellular α-tocopherol above the physiological level completely protected hepatocytes against the killing by allyl alcohol. In cells with physiological α-tocopherol, vitamin E declined within 30 min of exposure to allyl alcohol. This decrease paralleled the peroxidation of lipids, but preceded the decrease in cellular ascorbate. Under these conditions, a decline in ascorbate correlated with the loss of cell viability. Cells supplemented with at least 3 mM ascorbate prevented the decline in α-tocopherol. However, ascorbate acts as an independent antioxidant at these concentrations. In the absence of killing by allyl alcohol, the loss of cellular ascorbate did not depend on the presence or absence of cellular α-tocopherol. These data indicate that vitamins E and C act as separate antioxidants and that ascorbate does not regenerate the tocopheroxyl radical in cultured rat hepatocytes. BIOCHEM PHARMACOL 52;8:1245–1252, 1996.

KEY WORDS. allyl alcohol; vitamin E; vitamin C; cultured hepatocytes; oxidative stress; lipid peroxidation

α-Tocopherol (vitamin E) and ascorbate (vitamin C) are components of the antioxidant defenses of cells (reviewed Refs. 1–5). Vitamin E is found in biological membranes, where it is believed to react with and, thereby, detoxify lipid radicals [1, 2]. By contrast, vitamin C is a hydrophilic antioxidant that has two potential biological actions [3–5]. In addition to the direct reaction with lipid radicals [4–9], it has been proposed that vitamin C is also a reductant of the tocopheroxyl radical, an action that would regenerate the reduced form of vitamin E [5–7, 10–22].

We have examined previously the disposition of vitamins E and C in cultured hepatocytes subjected to an exogenous oxidative stress imposed by TBHP† [23, 24]. The underlying concern was to evaluate the nature of any interaction between these vitamins in living cells during the course of oxidative injury. The data indicated that vitamins E and C act as independent antioxidants and that vitamin C does not appear to act as a reductant of the tocopheroxyl radical in the intact cell. Vitamin C was an effective antioxidant in hepatocytes with either a physiological content of vitamin E or a virtual absence of this vitamin. In other words, the ability of vitamin C to protect liver cells from TBHP-induced cell killing did not depend on the vitamin E content of the cells. Additional evidence for the independence of the antioxidant action of α-tocopherol and ascorbate was the time course of the depletion of these vitamins in the hepatocytes undergoing oxidative injury [24]. A depletion of vitamin E preceded that of vitamin C, as well as the death of the cells. The loss of vitamin C was not observed before leakage of lactate dehydrogenase from the cells, a result that indicates that the decline in vitamin C is likely due to the loss of the integrity of the plasma membrane. Furthermore, total (reduced plus oxidized) ascorbate declined in the same manner as the reduced ascorbate content, a result arguing against recycling as a factor in the loss of reduced ascorbate.

In the present study, we have addressed the relevance of

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† *Abbreviations:* α-TP, α-tocopherol phosphate ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione; LDH, lactate dehydrogenase; MDA, malondialdehyde; SNK, Student–Neuman–Keuls *post hoc* test; and TBHP, *tert*-butyl hydroperoxide.

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these previous conclusions to a different model of oxidative liver cell injury. Rather than an exogenous, chemical oxidant, i.e. TBHP, we have examined the cellular disposition of vitamins E and C following the abrupt depletion of the GSH that is produced by exposure to allyl alcohol. Allyl alcohol is metabolized by alcohol dehydrogenase to produce the potent electrophile acrolein [25, 26] and, accordingly, results in periportal hepatic necrosis [27] in the intact rat. Acrolein reacts with and, thereby, abruptly depletes GSH in cultured rat hepatocytes [28], as well as in suspended hepatocytes [29–35] and in the isolated, perfused rat liver [36]. In cultured cells, lethal injury results from the peroxidation of membrane lipids produced by the constitutive flux of endogenous reactive oxygen species [28]. The data presented here support the hypothesis that vitamins E and C act independently to defend cells against oxidative stress. There was no evidence that ascorbate acts to reduce the tocopheroxyl radical in cultured hepatocytes undergoing lipid peroxidation induced by an endogenous oxidative stress.

MATERIALS AND METHODS

Hepatocytes

Male Sprague–Dawley rats (150–200 g) were obtained from Charles River (Wilmington, MA). The animals were housed for at least 1 week in quarters approved by the American Association for the Accreditation of Laboratory Animals Care, and allowed food (Purina Rodent Laboratory Chow 5001) and water *ad lib*. Rats were fasted overnight prior to use. Cultured hepatocytes were prepared by the method of Seglen [37]. The cells were plated at a density of 1.33×10^6 cells per 25 cm^2 polystyrene flask (Corning Costar Corp. Oneonta, NY) and then incubated overnight in Williams' E medium with 10% fetal bovine serum, insulin, gentamicin, penicillin, and streptomycin as previously described [23]. Additional incubation conditions were 37° in 95% air:5% CO_2.

Depending upon the experiment, a final concentration of 1–50 μM *dl*-α-tocopherol-phosphate ester, disodium (α-TP) (Sigma Chemical Co., St. Louis, MO) was added to or omitted from flasks during overnight incubation. α-TP was dissolved in water and added at 1% volume. Despite the fact that Williams' E medium with 10% serum was 18 nM α-TP and 43 ± 15 nM α-tocopherol, the vitamin E content of hepatocytes significantly declined after 16–18 hr in culture [23]. Supplementation with at least a 1 μM concentration of an α-tocopherol ester was required to maintain a physiological content of vitamin E [23]. In experiments involving vitamin C, a final concentration of 0.01 to 10 mM sodium ascorbate (Sigma) was dissolved in water and added to overnight flasks (1% volume). The ascorbate in Williams' E medium was undetectable 2 days after preparation, presumably due to auto-oxidation [24]. The medium used in these experiments was prepared at least a week prior to use. Both vitamin additions occurred after the first washing which was 2–3 hr after the plating. Then cells were incubated 16–18 hr as previously described [23], and all experiments were performed in these cells.

After 16–18 hr, flasks were washed twice with 3 mL of warm HEPES buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10.1 mM HEPES, pH 7.4). Williams' E medium without fetal bovine serum (serum-free medium) (5 mL) was added to cultures. It should be emphasized that the additional α-TP and ascorbate that were included in the overnight cultures were omitted from the medium during all experiments. Cells were again incubated under 95% air: 5% CO_2 at 37°. TBHP (Sigma) and allyl alcohol (Eastman Kodak) were diluted in serum-free medium and water, respectively, and added to the cultures (1% volume). The concentrations of TBHP and allyl alcohol as well as the times of sampling are indicated in Results and in the figure legends.

Ascorbate Determinations

Ascorbic acid (reduced form) and the internal standard tyrosine were determined by modification [24] of the method of Behrens and Madere [38]. Ascorbate was quantitated in the *m*-phosphoric acid supernatant of cultured cells by HPLC with a UV/visible spectrophotometric detector. A 4.6 × 150 mm Supelcosil LC-ABZ C_{18} column (5 μm packing) with an LC-ABZ C_{18} precolumn (Supelco Inc., Bellefonte, PA) was used. Sample preparation and conditions for HPLC analysis were also as previously described [24].

Tocopherol Determinations

Cellular α-tocopherol and the internal standard δ-tocopherol (Sigma) were determined by modification of the methods of Burton *et al.* [39] and Liebler *et al.* [12] by HPLC using fluorometric detection as described previously [23]. A 4.6 × 150 mm Supelcosil LC C_{18} column (3 μm packing) with a C_{18} precolumn (Supelco Inc.) was used. Sample preparation and conditions for HPLC analysis were also as previously described [23].

Alcohol and Aldehyde Dehydrogenases

The activities of both enzymes were assessed as the increased production of NADH at 340 nm for 5 min at room temperature. The activity of alcohol dehydrogenase was determined by a modification of the method of Crow *et al.* [40] as described by Penttilä [34]. Briefly, 100 μL of a 1% Triton X-100 homogenate of hepatocytes (approx. 0.5 mg protein) was incubated for 2–5 min with final concentrations of 500 mM Tris-HCl (pH 7.2) and 3 mM NAD^+. The reaction was initiated with a final concentration of 14 mM ethanol. The final volume was 1 mL. Tris efficiently binds the acetaldehyde formed from ethanol [34, 40].

The activity of aldehyde dehydrogenase was determined by a modification of the method of Mitchell and Petersen [41] as described by Silva and O'Brien [33]. Briefly, 100 μL of a 1% Triton X-100 homogenate of hepatocytes (approx.

0.5 mg protein) was incubated for 2–5 min with final concentrations of 100 mM sodium phosphate (pH 7.4), 3 mM NAD^+, and 2 mM pyrazole. The reaction was initiated with a final concentration of 5 mM glyceraldehyde. The final volume was 1 mL. Both enzyme activities were linear over the time measured, and an extinction coefficient of 6.22 mM was used for NADH.

Other Assays

The GSH in hepatocytes was determined spectrophotometrically using DTNB (Sigma) by a modification of the methods of Riddles *et al.* [42] and Jocelyn [43]. Briefly, cells were scraped into 2 mL of 5% trichloroacetic acid. A 400-μL aliquot of acid supernatant was added to 1 mL of a 600 mM potassium phosphate–1.5 mM EDTA buffer (pH 7.2). To this, 100 μL of a 3 mM solution of DTNB made in the phosphate–EDTA buffer was added. After 2 min, the absorbance was read at 412 nm.

Lipid peroxidation was assayed as the accumulation of thiobarbituric acid-reactive products in the culture medium (i.e. MDA) by a modification of the method of Ohkawa *et al.* [44] and was described previously [23]. Cell killing was determined by release of LDH into the medium [23]. The total amount of LDH was determined by treating a set of flasks with a final concentration of 0.5% Triton X-100. Protein was determined on each sample by the method of Smith *et al.* [45] (bicinchoninic acid) using BSA 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 is indicated in the figure legends. Data from flasks within a given experiment were considered paired since all the hepatocytes for one experiment came from a single rat. One- and two-way ANOVA using a repeated measures design and the SNK were performed on data using the PC version of an SAS statistical package (ver. 6.04) [46]. When unequal variance was encountered, the logarithmically transformed data were analyzed [47]. Data represent means ± SD.

RESULTS

Decreased Cellular α-Tocopherol in the Potentiation of Hepatotoxicity by Allyl Alcohol

The vitamin E content of hepatocytes decreases by 85% upon culturing the cells overnight in Williams' E medium [23, 24]. Supplementation of the medium with at least a 1 μM concentration of an α-tocopherol ester maintains a physiological content of vitamin E [23]. Figure 1 illustrates the effect of increasing concentrations of allyl alcohol on the vitamin E content (A) and the viability (B) of hepatocytes cultured 16–18 hr in the presence or absence of 1 μM α-TP. In α-TP-supplemented cells, allyl alcohol produced a concentration-dependent decline in the vitamin E content. At the same time, allyl alcohol produced a concentration-dependent increase in the extent of cell killing. With 4 mM allyl alcohol, vitamin E was depleted by 40%, and 35% of the hepatocytes were dead.

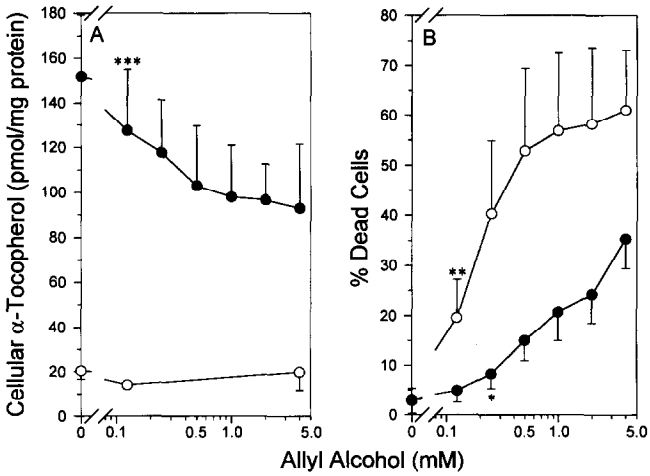

FIG. 1. Effect of 1 μM α-TP overnight supplementation on cellular α-tocopherol concentration in hepatocytes (A) and cell killing (B) after a 90-min exposure to 0, 0.125, 0.250, 0.500, 1, 2, and 4 mM allyl alcohol. Hepatocytes were prepared and incubated overnight with (●) or without (○) 1 μM α-TP. After 16–18 hr, cells were washed, placed in serum-free Williams' E medium without additional α-TP, and then incubated with allyl alcohol for 90 min. Values are the means ± SD for the results of 3–4 experiments. There was no change in the α-tocopherol content or indication of cell killing in untreated hepatocytes over the course of the experiment. Cells supplemented with α-TP showed greater cellular α-tocopherol ($P < 0.0001$ from ANOVA) and lower cell killing ($P < 0.02$ from ANOVA) than unsupplemented cells. Asterisks indicate the first point at which statistical differences were present: (*)$P < 0.05$ compared with respective zero group (from SNK); ($P < 0.01$ compared with respective zero group (from SNK); and (***)$P < 0.005$ compared with respective zero group (from SNK).**

Hepatocytes depleted of vitamin E following a 16 to 18-hr incubation without supplementation with α-TP were more sensitive to allyl alcohol (Fig. 1A). Maximal cell killing (55%) was achieved with 500 μM allyl alcohol. There was no measurable loss of vitamin E from the already depleted hepatocytes upon exposure to allyl alcohol (Fig. 1B).

Increased Cellular α-Tocopherol in the Protection against Hepatotoxicity by Allyl Alcohol

Figure 2 details the effect of supplementing the culture medium with α-TP on the cellular content of vitamin E prior to treatment with (A) and the resulting cell killing by (B) 2 mM allyl alcohol. Incubation with concentrations of α-TP from 1 to 50 μM increased the cellular content of vitamin E. In turn, hepatocytes supplemented with α-TP were less sensitive to killing by allyl alcohol than were hepatocytes not supplemented with α-TP. Cells supple-

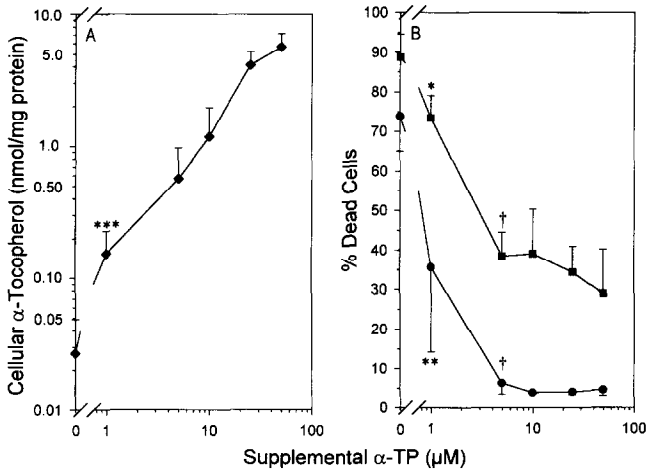

FIG. 2. Effect of increasing α-TP in the overnight medium on (A) the α-tocopherol concentrations and (B) cell killing in hepatocytes treated with 2 mM allyl alcohol (●) and 750 μM TBHP (■). Hepatocytes were prepared and incubated overnight with 0, 1, 5, 10, 25, or 50 μM α-TP. After 16–18 hr, cells were washed, placed in serum-free medium without α-TP, and then incubated with 2 mM allyl alcohol or 750 μM TBHP for 90 min. The α-tocopherol concentrations are the content prior to exposure to TBHP or allyl alcohol. Values are the means ± SD for the results of 5–6 experiments. Control cells not treated with allyl alcohol or TBHP showed no cell killing over the course of 90 min (data not shown). Asterisks and daggers indicate the first point at which statistical differences were present: (*)$P < 0.005$ from respective zero group (from SNK); (**)$P < 0.001$ from respective zero group (from SNK); (***)$P < 0.0001$ from respective zero group (from SNK); and (†)$P < 0.0001$ from respective 1 μM group (from SNK).

mented with 5 μM or greater α-TP were protected completely against the oxidative injury produced by allyl alcohol.

Data have shown previously that the killing of cultured hepatocytes by allyl alcohol is the consequence of the peroxidation of cellular phospholipids [28]. Thus, the protection by supplemental α-TP shown in Fig. 2 is readily explained by the well known action of vitamin E in preventing the peroxidation of lipids. By contrast, the killing of cultured hepatocytes by TBHP has been attributed to two distinct mechanisms of oxidative cell injury. In addition to lipid peroxidation [48], there is also a non-peroxidative mechanism of lethal cell injury that was related previously to the loss of mitochondrial energization [49].

Figure 2B also details the effect of supplemental α-TP on the killing of cultured hepatocytes by TBHP. Supplemental α-TP reduced but did not completely prevent the cell killing by TBHP. Interestingly, the concentration–response relationship between supplemental α-TP and cell killing parallels that of the protection against the toxicity of allyl alcohol. Maximum protection against the toxicity of both TBHP and allyl alcohol was achieved with 5 μM α-TP. The fact that higher concentrations of α-TP did not further reduce the cell killing by TBHP is explained by the inability of lipophilic antioxidants to protect against the non-peroxidative mechanism of lethal cell injury [23, 48, 49].

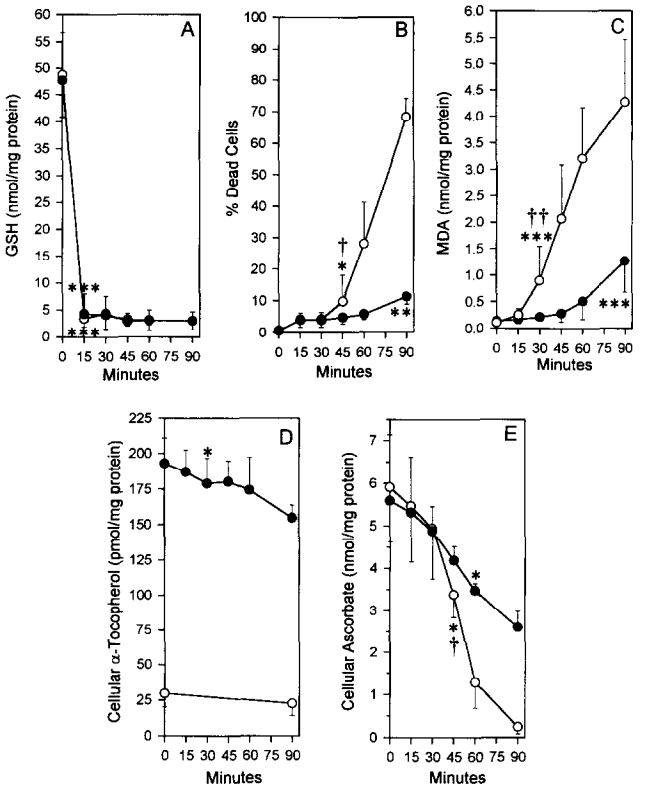

FIG. 3. Effect of 350 μM allyl alcohol on hepatocytes incubated overnight with or without 1 μM α-TP. Hepatocytes were prepared and incubated overnight with (●) or without (○) 1 μM α-TP. After 16–18 hr, cells were washed, placed in serum-free medium without additional α-TP, and then incubated with allyl alcohol. (A) GSH content, (B) cell viability, (C) lipid peroxidation, (D) α-tocopherol content, and (E) ascorbate content were determined at the specified times. Values are the means ± SD for the results of 4–6 experiments. Control cells not treated with allyl alcohol showed no changes in any of the measurements over the course of 90 min (data not shown). Asterisks and daggers indicate the first point at which statistical differences were present: (*)$P < 0.05$ from respective zero time; (**)$P < 0.005$ from respective zero time; (***)$P < 0.001$ from respective zero time; (†)$P < 0.05$ from same time in α-TP-supplemented cells; and (††)$P < 0.001$ from same time in α-TP-supplemented cells.

Cellular Content of α-Tocopherol and Ascorbate Following Exposure to 350 μM Allyl Alcohol

Hepatocytes were cultured for 16–18 hr in the presence or absence of 1 μM α-TP. The cells were washed and placed in serum-free medium without vitamin supplementation. The cells were then treated with 350 μM allyl alcohol. Figure 3 illustrates the time course of the resulting changes in the content of GSH (A), cell viability (B), lipid peroxidation (C), vitamin E (D), and vitamin C (E).

Allyl alcohol is metabolized to acrolein by alcohol dehydrogenase. Acrolein is a potent electrophile that depletes the hepatocytes of GSH. Figure 3A shows that there was no effect of supplementation with α-TP on the rate or extent of the depletion of GSH by 350 μM allyl alcohol. By contrast, less than 10% of the hepatocytes supplemented with

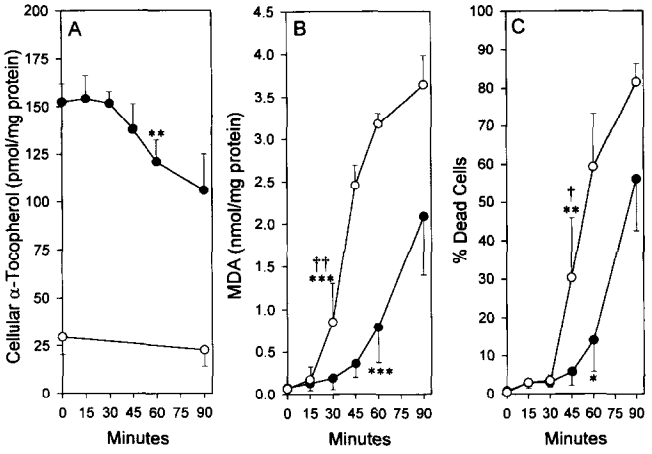

FIG. 4. Effect of 2 mM allyl alcohol on (A) cellular α-tocopherol concentrations, (B) lipid peroxidation, and (C) cell killing after 90 min. Hepatocytes were prepared and incubated overnight with (●) and without (○) 1 μM α-TP. After 16–18 hr, cells were washed and placed in serum-free medium without α-TP. The cells were then incubated with 2 mM allyl alcohol for 90 min. Values are the means ± SD for the results of 4–5 experiments. Cells not treated with allyl alcohol showed no changes in any of the measurements over the course of 90 min (data not shown). Asterisks and daggers indicate the first point at which statistical differences were present: (*)$P < 0.01$ from respective zero time; (**)$P < 0.001$ from respective zero time; (***)$P < 0.0001$ from respective zero time; (†)$P < 0.001$ from the same time in α-TP-supplemented cells; and (††)$P < 0.0001$ from the same time in α-TP-supplemented cells.

α-TP died over the 90-min course of the experiment (Fig. 3B), whereas almost 70% of unsupplemented cells died over the same time period. Lipid peroxidation, as demonstrated by the accumulation of MDA in the medium (Fig. 3C), occurred to a much greater extent in the unsupplemented cultures and was detectable prior to the release of LDH. MDA accumulation was significant only after 90 min in the supplemented cultures and correlated with the minimal cell killing in Fig. 3B.

In supplemented hepatocytes, the cellular content of vitamin E (Fig.3D) declined only slightly, a result consistent with the minimal lipid peroxidation (Fig. 3C) and cell killing (Fig. 3B). Unsupplemented hepatocytes contained approximately 15% of the initial vitamin E concentrations and showed no change over the 90-min course of the experiment (Fig. 3D). Cellular ascorbate concentrations in α-TP-supplemented hepatocytes did not differ from unsupplemented hepatocytes until 45 min after exposure to allyl alcohol (Fig. 3E). There was no significant decline in the ascorbate content until 45 min in the unsupplemented hepatocytes and until 60 min in the supplemented hepatocytes. After these times, the ascorbate continued to decline in both groups, although at a faster rate in the unsupplemented cells. By 90 min, the ascorbate content of the hepatocytes decreased by 53% in vitamin E-supplemented cells and by 95% in the unsupplemented cells. The decline in ascorbate (Fig. 3E) paralleled the increase in cell killing (Fig. 3B).

In an ancillary experiment, hepatocytes were cultured for 16–18 hr with 2 mM ascorbate and in the presence or absence of 1 μM α-TP. Cells treated with this concentration of ascorbate contained approximately 44 nmol of vitamin C/mg protein. Treatment of the cells with 350 μM allyl alcohol produced a 20% decline in the cellular ascorbate by 30–45 min, a decrease that did not depend on the presence or absence of vitamin E (data not shown). This decline was corrected for the release of ascorbate into the medium at a rate of 8.16 nmol/mg protein/hr which occurred in these hepatocytes (data not shown). In addition, the ascorbate supplementation did not affect the abrupt decline in GSH caused by allyl alcohol but did completely protect the cells against killing by this dose of allyl alcohol (data not shown).

Cellular Content of α-Tocopherol Following Exposure to 2 mM Allyl Alcohol

Higher concentrations of allyl alcohol than were used in the previous experiments more readily documented the relationship between vitamin E content, lipid peroxidation, and cell killing with oxidative stress. In α-TP-supplemented hepatocytes, the cellular content of vitamin E declined by almost a third within 90 min of exposure to 2 mM allyl alcohol (Fig. 4A). The greater loss of vitamin E with the larger dose of allyl alcohol was accompanied by a more substantial accumulation of MDA (Fig. 4B) and greater loss of viability (Fig. 4C). Importantly, unsupplemented hepatocytes reacted to the higher dose of allyl alcohol (Fig. 4, A–C) in a manner very similar to that seen with the lower dose (Fig. 3, B–D).

Sparing of Cellular α-Tocopherol by Increased Cellular Ascorbate during Exposure to Allyl Alcohol

Hepatocytes were cultured overnight with 0.01 to 10 mM ascorbate in the presence or absence of 1 μM α-TP. The next day the cells were washed and placed in fresh medium without either vitamin. Figure 5 illustrates the effect of ascorbate supplementation on the cell killing by 2 mM allyl alcohol, as well as the effect on the cellular content of vitamin E.

Supplementation with ascorbate did not affect the loss of cellular α-tocopherol that occurs after 16–18 hr in culture (data not shown). Nevertheless, incremental concentrations of ascorbate in the overnight culture medium increasingly protected against the cell killing with 2 mM allyl alcohol (Fig. 5A). Hepatocytes supplemented with α-TP were killed by 2 mM allyl alcohol to a lesser extent than unsupplemented cells. In addition, increasing concentrations of ascorbate in the overnight culture medium further reduced the toxicity of 2 mM ally alcohol. With 0.1 mM and higher ascorbate, there was complete protection. This protection was not explained by any effect of the ascorbate supplementation on the rate or extent of the depletion of GSH produced by allyl alcohol (data not shown).

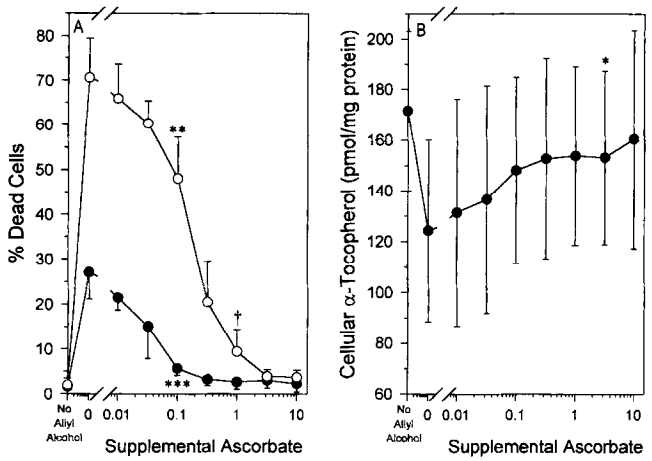

FIG. 5. Effect of increasing overnight supplementation with ascorbate on (A) cell killing and (B) cellular α-tocopherol concentration in hepatocytes after a 90-min exposure to 2 mM allyl alcohol. Hepatocytes were prepared and incubated overnight with (●) or without (○) 1 μM α-TP and with 0, 0.010, 0.032, 0.10, 0.32, 1.0, 3.2, or 10 mM ascorbate. After 16–18 hr, cells were washed, placed in serum-free Williams' E medium without vitamin supplementation, and then incubated with 2 mM allyl alcohol for 90 min. Values are the means ± SD for the results of 4 experiments. Asterisks and daggers indicate the first point at which statistical differences were present: (*)$P < 0.05$ compared with no allyl alcohol group (from SNK); (**)$P < 0.0005$ compared with respective zero supplementation group (from SNK); (***)$P < 0.0001$ compared with respective zero supplementation group (from SNK); and (†)$P < 0.05$ from the same ascorbate supplementation in α-TP-supplemented cells.

Allyl alcohol reduced the cellular content of vitamin E in hepatocytes supplemented overnight with α-TP (Fig. 5B). (This result is essentially the same as that shown in Fig. 4A.) Incremental concentrations of ascorbate in the overnight culture medium along with α-TP increasingly protected against the loss of vitamin E upon treatment with 2 mM allyl alcohol (Fig. 5B). With concentrations of ≥3 mM ascorbate, the content of α-tocopherol was not significantly lower than that in untreated cells. It should also be noted that neither supplementation to maintain normal vitamin E content nor supplementation to achieve supraphysiological contents of vitamin E and/or vitamin C had any effect on the activities of alcohol and aldehyde dehydrogenases in cultured hepatocytes (data not shown).

DISCUSSION

The present study details the metabolism of vitamins E and C in cultured hepatocytes injured by the constitutive flux of activated oxygen species. A susceptibility to endogenous oxidants resulted from the abrupt depletion of GSH produced, in turn, by the metabolism of allyl alcohol [28]. With this hepatotoxin, peroxidation of cellular lipids follows the loss of GSH produced by acrolein, the electrophilic metabolite of allyl alcohol. The peroxidation of phospholipids destroys the permeability barrier function of the plasma membrane, an event that determines the death of the cells. This mechanism of oxidative injury allowed an evaluation of the interaction of vitamins E and C in intact cells under differing conditions than those studied previously by our laboratory [24].

We evaluated the ability of vitamins E and C to prevent the oxidative injury of cultured hepatocytes under conditions where the cellular content of each vitamin varied substantially. Hepatocytes maintain a constant content of ascorbate during the course of a day in culture [24], and supplementation of the medium for 16–18 hr can raise this content over an order of magnitude. By contrast, the cellular content of vitamin E decreases by 85% after 16–18 hr in culture. Supplementation of the medium with α-TP maintains the physiologic content of vitamin E without any effect on the ascorbate content of the cells [24]. Furthermore, the vitamin E content of the cells can be increased above the physiologic level with greater supplementations of α-TP. Thus, the intracellular content of vitamins E and C can be manipulated independently over a wide range. In turn, the effect of such manipulations on the sensitivity of the hepatocytes to allyl alcohol was evaluated. We again mention that all experiments were performed in the absence of exogenous vitamin supplementation.

The sensitivity of the cultured hepatocytes to allyl alcohol varied with the vitamin E content of the cells (Figs. 1 and 2). Furthermore, this variation occurred in the presence of a constant physiological content of vitamin C. A reduced cell killing paralleled the decrease in the peroxidation of cellular lipids seen with increased vitamin E (Fig. 3). A loss of vitamin E preceded the accumulation of MDA and, thus, the death of the cells. In hepatocytes depleted of vitamin E, the ascorbate content of the cells did not change until the release of LDH (Fig. 3). More importantly, there was no change in the ascorbate content prior to the loss of vitamin E in the hepatocytes that were not depleted of this vitamin E, a result that would not support a role for vitamin C in maintaining vitamin E levels.

Higher doses of allyl alcohol than those used in the studies detailed in Figs. 1 and 3 confirmed the relationship between vitamin E, lipid peroxidation, and lethal cell injury. When vitamin E-supplemented hepatocytes were treated with 2 mM allyl alcohol (Fig. 4), depletion of vitamin E accompanied the accumulation of MDA in the culture medium, as well as the release of LDH. Increasing the cellular content of ascorbate in α-TP-supplemented hepatocytes reduced the extent of cell killing and spared the depletion of vitamin E (Fig. 5). However, this action of vitamin C reflects its independent antioxidant action, as there is no significant loss of vitamin C prior to the decline of vitamin E. High concentrations of ascorbate do not protect the cells by preventing the depletion of GSH. The rates and extent of GSH decline caused by allyl alcohol are the same in cells with physiological and pharmacological contents of vitamin C.

The present data support those of others using living models which indicate no role for vitamin C in the regeneration of vitamin E. Burton *et al.* [50] studied the uptake and loss of radiolabeled vitamin E in nine tissues in guinea pigs receiving diets with high, normal, or low vitamin E and/or high, normal, or low vitamin C contents. They determined that the sparing of vitamin E was negligible when compared to the metabolic process that utilizes vitamin E. Others have shown a role for vitamin C, independent from that of vitamin E, in protection against several toxins [29]. The latter study was performed in isolated hepatocytes soon after collagenase perfusion and with the continued presence of supplemental ascorbate in the incubation medium. In addition, 4 weeks on a special diet was required to obtain vitamin E-deficient rats which could then be used in experiments. Culturing of hepatocytes allows the cells to recover from the disruption of the isolation procedure. As mentioned, the content of α-tocopherol and ascorbate in the present study can be manipulated easily during 16–18 hr in culture, and the experiments were performed without addition of exogenous vitamins to the culture medium.

Unlike the findings above, others have claimed interaction between vitamins E and C. Most if not all of these studies were done in static, non-living systems [5–7, 10–22]. While the regeneration of the tocopheroxyl radical by ascorbate can be demonstrated in these models, the question of whether this interaction occurs in an intact cell under physiological conditions remained. Experiments in whole animals also have claimed interaction. ODS rats are mutant rats that contain a missense mutation in the enzyme gulonolactone oxidase and thus cannot synthesize ascorbate [51, 52]. When fed diets enriched in ascorbate, ODS rats had greater tissues concentrations of vitamin E [53]. However, the scavenaging of endogenous radicals by ascorbate, rather than regeneration of the tocopheroxyl radical by ascorbate, can easily explain the higher vitamin E content observed in these animals.

It is clear that the data derived from the present study of the disposition of vitamins E and C during the course of oxidative liver cell injury produced by allyl alcohol support the previously reported conclusions based on the analysis of the killing of cultured hepatocytes by the exogenous oxidant TBHP. Thus, when liver cells are injured by oxidants imposed on the cells from the culture medium or derived from the constitutive flux of activated oxygen, vitamins E and C act as independent antioxidants. In other words, the cellular content of vitamin E does not affect the action or content of vitamin C. Stated differently, there is no evidence that ascorbate acts as a reductant of the tocopheroxyl radical. Whereas pharmacological concentrations of vitamin C do spare the depletion of vitamin E during oxidative injury, the simplest interpretation of this action of ascorbate is that it reflects the direct antioxidant action of this vitamin. By detoxifying the radicals that initiate and/or propagate lipid peroxidation, vitamin C reduces the participation of vitamin E in the control of oxidative membrane damage.

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