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The Antioxidant Function of the Physiological Content of Vitamin C

PETER A. GLASCOTT, JR., MARIYA TSYGANSKAYA, ELLEN GILFOR, MARK A. ZERN, and JOHN L. FARBER

From the Departments of Pathology (P.A.G., E.G., J.L.F.) and Medicine (M.T., M.A.Z.), Division of Gastroenterology and Hepatology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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SUMMARY

The synthesis of vitamin C is substantially reduced in Osteogenic Disorder Shionogi (ODS) rats. Hepatocytes prepared from these rats contained ~12% of the wild-type content of this vitamin. In culture, the ascorbate content remained low in the absence of supplementation of the medium. Independent of their vitamin C status, cultured hepatocytes become depleted of vitamin E. Supplementation of the culture medium with 100 μM ascorbate and 1.2 μM α-tocopherol phosphate maintained the physiological content of both vitamins C and E in ODS hepatocytes. Thus, the antioxidant function of vitamins C and E could be evaluated in the presence of both or either vitamin or in the absence of both vitamins. Hepatocytes deficient in both vitamins were the most susceptible to lipid peroxidation (as measured by thiobarbituric acid) and to cell killing within a 90-min exposure to 125–500 μM *tert*-butyl hydroperoxide (TBHP). Supplementation to achieve a physiological content of both vitamins C and E reduced the evidence of lipid peroxidation and abolished the cell killing. Supplementation with either vitamin alone resulted in an intermediate degree of both lipid peroxidation and cell killing. In ODS hepatocytes treated with TBHP, the decline in vitamin E preceded the decline in vitamin C. In ODS hepatocytes depleted of vitamin C, the loss of vitamin E after exposure to TBHP was greater than that in the presence of physiological levels of ascorbate. This greater loss of vitamin E in the face of a depletion of vitamin C was readily attributable to the increased peroxidation of lipids. Thus, the physiological level of vitamin C in cells does not seem to regenerate vitamin E. In contrast, the rate and extent of the depletion of vitamin C correlate with the degree of cell killing. These data document the antioxidant function of the physiological level of cellular vitamin C and relate this function to protection against peroxidative cell injury.

Ascorbate (vitamin C) is a water-soluble antioxidant that is widely thought to participate in the antioxidant defense of cells (for reviews, see Refs. 1–3). A variety of model systems document the protective action of this vitamin against oxidative injury after exposure to a number of different hazards (4–11). Two separate mechanisms can account for this protective action. On the one hand, it has been suggested that vitamin C reduces the tocopheroxyl radical to α-tocopherol (vitamin E), a reaction that regenerates the active antioxidant form of vitamin E (1, 2, 7, 10–18). Vitamin E is the major antioxidant in biological membranes (for reviews, see Refs. 12 and 13). Alternatively, vitamin C has been shown to react directly with oxygen radicals (1, 2, 9, 10, 19, 20), an effect that is independent of any relationship to the metabolism of vitamin E.

Recent studies from this laboratory using cultured hepatocytes addressed the question of the interaction of vitamins E and C (4, 5). TBHP is widely used to model the more physiological oxidative stress imposed by hydrogen peroxide. In this regard, similar concentrations of TBHP (21) and hydrogen peroxide (22) kill comparable numbers of cultured hepatocytes. Although the antioxidant properties of supplemental vitamin C was readily demonstrable, there was no evidence that it acted as a reductant of the vitamin E radical (4, 5). In other words, vitamin C seemed to function as a parallel antioxidant defense, independent of vitamin E. This conclusion was based on data derived from two sets of experiments. First, the ability of supplemental vitamin C to protect cultured hepatocytes from oxidative killing by TBHP did not depend on the vitamin E content of these cells; that is to say, supplemental vitamin C protected to the same extent in cells with a physiological content of vitamin E or a substantial depletion of this vitamin. Second, there was no loss of reduced vitamin C before the loss of vitamin E.

Although this previous study argued that increased cellular vitamin C acts as an antioxidant independent of vitamin E, the question of the antioxidant action of the physiological

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ABBREVIATIONS: α-TP, α-tocopherol phosphate ester; GSH, glutathione; ANOVA, analysis of variance; MDA, malondialdehyde; ODS, Osteogenic Disorder Shionogi; TBHP, *tert*-butyl hydroperoxide; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

content of this vitamin was not resolved. Rat liver cells synthesize vitamin C in both the intact animal and culture. Thus, it was not possible to deplete the cultured hepatocytes of ascorbate and, accordingly, to assess the antioxidant action of the physiological content of this vitamin. The ODS strain of rats is unable to synthesize vitamin C (18, 23, 24) due to a missense mutation (25) in the gene encoding L-gulono-γ-lactone oxidase, the last enzyme in the biosynthetic pathway of ascorbate. This same gene is inactive in humans, primates, and guinea pigs, species that do not synthesize vitamin C (26–28). In the current study, cultured hepatocytes were prepared from ODS rats. These hepatocytes contained ~12% of the normal content of vitamin C and were used to assess the antioxidant function of the physiological content of this vitamin. Furthermore, ODS hepatocytes lose >85% of their initial content of vitamin E after 16–18 hr in culture, a loss almost identical to that observed in normal hepatocytes that are cultured. The data demonstrate that the physiological content of vitamin C serves as an independent antioxidant that protects against cell injury and does not seem to regenerate vitamin E.

Materials and Methods

Hepatocytes. Male ODS rats (4 weeks old) were obtained from Clea (Tokyo, Japan). For ≥2 weeks before use, the animals were maintained in housing approved by the American Association for the Accreditation of Laboratory Animal Care and allowed food (Purina Rodent Laboratory Chow 5001) and water *ad libitum*. This brand of rat chow contains 40 IU/kg vitamin E and no ascorbate. Clinical symptoms of scurvy (29) were largely prevented by supplementing the drinking water with 200 mg/liter Ester C (72% calcium ascorbate, 9% dehydroascorbate, and 1% calcium threonate; Inter-Cal, Prescott, AZ). Occasionally, when rats became scorbutic, additional supplementation with sodium ascorbate (100 mg/liter in the water and/or by dusting the food pellets) alleviated the symptoms. ODS rats on this regimen for 14 weeks gained weight at a rate comparable to wild-type rats (data not shown). Rats were fasted overnight before use. Ester C water was not removed before preparation of hepatocytes. Isolated hepatocytes were prepared according to the method of Seglen (30) and were comparable in viability (90–95%) to hepatocytes from Sprague-Dawley rats. Cells were plated onto 25-cm^2 polystyrene flasks (Corning Costar, Cambridge, MA) at a density of 1.33×10^6 cells/flask in 3 ml of Williams' E medium (GIBCO, 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 (JHR 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 warm HEPES buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10.1 mM HEPES, pH 7.4, 37°) to remove any unattached or dead cells. Five milliliters of fresh complete Williams' E medium was then added, and the hepatocytes were incubated overnight. Depending on the treatment group, a final concentration of 1.2 μM DL-α-tocopherol phosphate, disodium (Sigma Chemical, St. Louis, MO) and/or 100 μM sodium ascorbate (Sigma) was added to or omitted from flasks during overnight incubation. Both vitamins were added at 1% volume in water.

Overnight cultures (16–18 hr) were washed twice with 3 ml of warm HEPES buffer. Five milliliters of complete Williams' E medium without fetal calf serum (serum-free Williams' E medium) was added to cultures. It should be emphasized that the additional α-TP and/or ascorbate added to the overnight cultures was omitted from the medium during all experiments. Cells were incubated under 5% CO_2/95% air at 37°. TBHP (Sigma) was diluted in serum-free medium and added to the cultures (1% volume). The concentrations of TBHP and times of sampling are indicated in Results and figure legends. After incubations, cells were gently washed with HEPES buffer, and ascorbate or α-tocopherol levels were determined as described below.

Ascorbate determinations. Cellular ascorbic acid (reduced form) levels were determined by modification (4) of the method of Behrens and Madere (31) using tyrosine as an internal standard (32). After washing with HEPES buffer, the cellular ascorbate was quantified in the *m*-phosphoric acid supernatant 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, Bellefonte, PA) was used. Sample preparation and conditions for the HPLC analysis were described previously (4).

α-Tocopherol determinations. Cellular α-tocopherol and the internal standard α-tocopherol (Sigma) were determined through modification of the methods of Burton *et al.* (33) and Liebler *et al.* (14) by HPLC and fluorometric detection as described previously (21). After washing with HEPES buffer, the cells were scraped into 2 ml of 50% ethanol with 0.1 mg/ml butylated hydroxytoluene. The samples, along with a standard curve, were stored overnight at −70° and analyzed the next day.

Other assays. Lipid peroxidation was assayed as the accumulation of thiobarbituric acid-reactive products in the medium through a modification of the method of Ohkawa *et al.* (34) and as described previously (4, 21). The death of the cells was determined by release of lactate dehydrogenase into the medium (21). The GSH content of hepatocytes was determined spectrophotometrically using 5,5′-dithio-bis-2-nitrobenzoic acid according to a modification of the methods of Riddles *et al.* (35) and Jocelyn (36). Protein was determined on each sample by using bicinchoninic acid (37) with bovine serum albumin as the standard.

Statistical analysis. Data from duplicate 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 because all of the hepatocytes for one experiment came from a single rat. One- and two-way ANOVAs using a repeated-measures design and Student-Neuman-Keuls post hoc tests were performed on data using the PC version of SAS statistical software (version 6.04; SAS Institute, Cary, NC) (38). When unequal variance was encountered, the logarithmically transformed data were analyzed (39). All data presented represent the mean ± standard deviation.

Results

Vitamin C, vitamin E, and GSH concentrations in ODS hepatocytes. It was first necessary to characterize the content of cellular ascorbate and α-tocopherol of ODS hepatocytes before their treatment with TBHP. Table 1 details these antioxidants, as well as GSH, before and after culturing of the isolated hepatocytes. Despite the absence of symptoms of scurvy in the intact rat, the reduced ascorbate content of hepatocytes freshly isolated from ODS rats was invariably 88% lower than that of hepatocytes prepared from nonmutant rats (i.e., 4.9 nmol/mg protein; Ref. 4). This low content of vitamin C did not change after 16–18 hr in culture. The addition of 100 μM ascorbate to the medium increased the cellular content of reduced ascorbate to a physiological level. Maintenance of the vitamin E levels with supplemental α-TP did not influence the ascorbate content of the cells. However, when ascorbate-supplemented cells were placed for 90 min in fresh serum-free medium devoid of ascorbate, the cellular content of vitamin C promptly declined, a result attributable, at least in part, to the release of ascorbate into the medium (data not shown). A more-detailed study demon-

TABLE 1

Characterization of the endogenous antioxidant concentrations in suspended and cultured hepatocytes prepared from ODS rats

Hepatocytes from ODS rats were isolated and cultured overnight in 5 ml of complete Williams' E medium in 5% CO_2/95% air at 37°. Cells were cultured in the presence or absence of 1.2 μM α-TP and/or 100 μM sodium ascorbate. On the next day, the cells were washed with warm HEPES buffer and placed in 5 ml of serum-free medium without vitamin supplementation. No TBHP was added to the flasks. Vitamin C, vitamin E, and GSH were assayed as described in Materials and Methods immediately after washing and 90 min after the addition of serum-free medium. Values are mean ± standard deviation. The number of determinations is indicated in parentheses.

		Ascorbate		α-TP		GSH	
		nmol/mg of protein		*pmol/mg of protein*		*nmol/mg of protein*	
Freshly isolated hepatocytes		0.6 ± 0.5 (6)		131 ± 23[a] (7)		18.3 ± 9.7[b] (5)	
Cultured hepatocytes (16–18 hr with)							
α-TP	Ascorbate	0 min[c,d]	90 min[c]	0 min[c,e]	90 min[e]	0 min[c]	90 min[f]
		nmol/mg of protein		*pmol/mg of protein*		*nmol/mg of protein*	
−	−	0.8 ± 0.7 (4)	0.9 ± 0.7 (3)	16 ± 2 (3)	10 ± 5 (3)	33.9 ± 8.5 (4)	44.9 ± 13.6 (3)
+	−	0.5 ± 0.4 (4)	0.7 ± 0.6 (3)	154 ± 35 (6)	206 ± 23 (3)	33.1 ± 9.1 (4)	42.4 ± 9.1 (3)
−	+	13.4 ± 0.7 (7)	8.2 ± 0.7 (4)	22 ± 2 (3)	15 ± 12 (3)	39.5 ± 9.3 (7)	41.0 ± 8.6 (4)
+	+	13.0 ± 1.4 (7)	8.0 ± 0.7 (4)	166 ± 32 (6)	204 ± 12 (3)	40.0 ± 9.2 (7)	42.9 ± 9.5 (4)

[a] Not significantly different from 0-min cultured hepatocytes supplemented 16–18 hr with α-TP.
[b] Significantly less than cultured hepatocytes regardless of vitamin supplementation ($p < 0.0001$, one-way ANOVA).
[c] Cells supplemented with ascorbate are significantly greater than cells not supplemented with ascorbate ($p < 0.05$, two-way ANOVAs).
[d] 0-min cells supplemented with ascorbate are significantly greater than 90-min cells supplemented with ascorbate ($p < 0.003$, two-way ANOVA).
[e] Cells supplemented with α-TP are significantly greater than cells not supplemented with α-TP ($p < 0.003$, two-way ANOVAs).
[f] Significantly greater than 0 min ($p < 0.03$, two-way ANOVAs.

strated that in cultured hepatocytes supplemented with ascorbate alone, the cellular ascorbate content declined at a rate of 3.48 nmol/mg of protein/hr (r^2 = 0.91; data not shown).

Cellular vitamin E concentrations in ODS hepatocytes declined by almost 90% after 16–18 hr in culture without α-TP supplementation (Table 1), a result similar to that seen with Sprague-Dawley hepatocytes (4, 21). Supplementation of the cultures with 1.2 μM α-TP maintained the α-tocopherol concentrations. There was no significant change in the vitamin E content at 90 min after the addition of fresh medium. Finally, cultures treated with 100 μM ascorbate had slightly higher vitamin E contents compared with cultures not incubated with ascorbate. However, this difference was not present after a 90-min incubation in medium without either vitamin.

The third endogenous antioxidant examined was GSH. After 16–18 hr in culture, the GSH content of ODS hepatocytes increased relative to freshly isolated cells. Hepatocytes supplemented with ascorbate had slightly higher GSH concentrations, and the GSH content increased further 90 min after the addition of fresh medium. Supplemental α-TP had no effect on the GSH content of the cultured hepatocytes. These changes in GSH content are similar to those reported with nonmutant hepatocytes (40).

Antioxidant action of vitamins C and E. ODS hepatocytes were cultured for 16–18 hr in the presence or absence of 1.2 μM α-TP and/or 100 μM ascorbate. The cells were then washed, placed in fresh medium without either vitamin, and exposed to increasing concentrations of TBHP for 90 min. Fig. 1 details the resulting changes in the extent of cell killing, the content of vitamin E, and the degree of lipid peroxidation. Ascorbate is a cytosolic component that protects cultured hepatocytes against cell killing by TBHP (4) and allyl alcohol (5). After treatment with either toxin, the decline in cellular ascorbate corresponded to the degree of cell killing. Thus, the content of cellular ascorbate in response to increasing concentrations of TBHP was not measured at the end of 90 min.

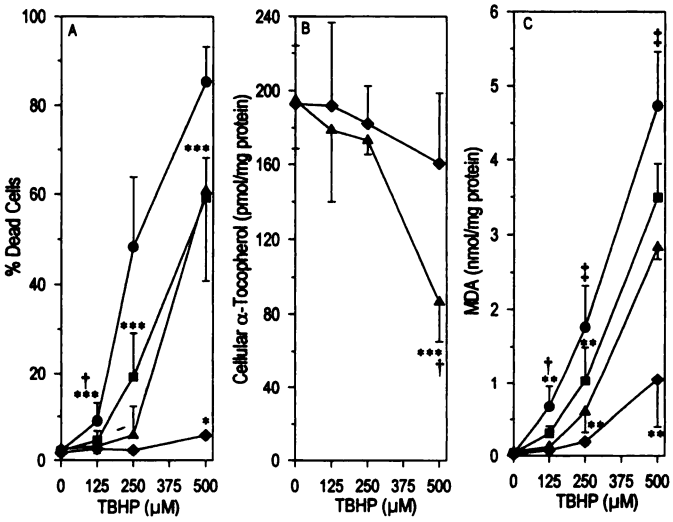

Fig. 1. Effect of TBHP concentration on cell killing (A), cellular vitamin E concentrations (B), and accumulation of MDA (C) in the medium of hepatocytes that were cultured overnight without α-TP and ascorbate (●), with either α-TP (▲) or ascorbate (■) alone, or with both vitamins (◆). Hepatocytes were prepared and incubated overnight with or without 1.2 μM α-TP and/or 100 μM ascorbate. After 16–18 hr, cells were washed and placed in Williams' E medium without serum and additional α-TP and/or ascorbate. The cells were then incubated with 0–500 μM TBHP for 90 min. *Asterisks* and *daggers*, first points at which statistical differences are present. Values are mean ± standard deviation for three experiments. *, $p < 0.05$ compared with respective no-TBHP treatment group. **, $p < 0.005$ compared with respective no-TBHP treatment group. ***, $p < 0.001$ compared with respective no-TBHP treatment group. †, $p < 0.05$ compared with other supplementation groups at the same concentration of TBHP. ††, $p < 0.05$; all supplementation groups are different from each other at the same concentration of TBHP.

There was essentially no cell killing in cultures supplemented with both ascorbate and α-TP (Fig. 1A). In contrast, in cultures that received neither vitamin, 500 μM TBHP killed >80% of the hepatocytes. Increased cell killing in the unsupplemented hepatocytes was evident with every dose of TBHP. Hepatocytes supplemented with either ascorbate or

α-TP alone were killed to an intermediate extent, a result that is most evident with 500 μM TBHP. Supplementation with either α-TP or ascorbate protected the cells to the same extent, but the cell killing was greater than in the group treated with both vitamins.

The content of vitamin E (Fig. 1B) paralleled the changes in cell killing and accumulation of MDA in the culture medium. In cells supplemented with both vitamins, there was no significant loss of vitamin E with any of the doses of TBHP. In contrast, in cultures supplemented with α-TP alone, 500 μM TBHP caused a 56% decline in the vitamin E content of the cells, a result that reflected the increased sensitivity of the hepatocytes to TBHP (Fig. 1A). The minimal level of vitamin E in cultured cells not supplemented with α-TP does not change over the course of the experiment, nor was this level decreased further after treatment with TBHP (4, 21).

Similarly, the differences in the extent of lipid peroxidation as measured by the accumulation of MDA in the culture medium reflected differences in the cell killing (Fig. 1C). The hepatocytes that were not supplemented with either ascorbate or α-TP showed the greatest accumulation of MDA, whereas those supplemented with both vitamins showed the least accumulation of MDA. Cultures supplemented with either α-TP or ascorbate alone accumulated an intermediate amount of MDA. Cells not treated with TBHP showed no increase in the MDA over the course of the experiment (data not shown).

Time course of antioxidant utilization during exposure to TBHP. Again, hepatocytes were cultured for 16–18 hr in the presence or absence of 1.2 μM α-TP and/or 100 μM ascorbate. The cells were then washed and placed in serum-free medium without any vitamin supplementation. Fig. 2 details the time course of changes in cell viability, content of vitamin E, content of vitamin C, and accumulation of MDA after treatment with 500 μM TBHP. This concentration of TBHP was chosen based on its effect on the vitamin E content in the cells in Fig. 1B.

Without any vitamin supplementation, cell killing was evident within 30 min. The number of dead cells increased over the next 30 min, with almost 80% of the cells killed at 1 hr. In hepatocytes supplemented with either ascorbate or α-TP alone, dead cells were also evident by 30 min, although to a lesser extent than without any supplementation. This trend persisted throughout the remaining 30 min of the experiment. Importantly, there was no significant difference in the extent of cell killing with either vitamin alone. Again, there was essentially no cell killing on supplementation with both vitamins.

In cells supplemented with α-TP alone, cellular vitamin E declined by almost 50% in the first 15 min and then remained at this level for the duration of the experiment (Fig. 2B). Hepatocytes supplemented with both α-TP and ascorbate showed only a 20% decline in the vitamin E content within 15 min, and there was no further loss over the subsequent 45 min. As mentioned previously, the minimal level of vitamin E in cultured cells not supplemented with α-TP did not change over the course of the experiment, nor was this level decreased further by treatment with TBHP (4, 21).

In contrast to the changes in vitamin E, cellular vitamin C content in ascorbate-supplemented hepatocytes did not differ during the first 15 min after exposure to TBHP, regardless of whether the cells were supplemented with α-TP (Fig. 2C). In hepatocytes containing ascorbate alone, the loss of vitamin C occurred only after the loss of viability seen in Fig. 2A. That is, cellular ascorbate content was decreased by 45 min, which is 15 min after cell death was first evident. Ascorbate declined further by 60 min and corresponded to the degree of cell killing. This trend was also suggested in cells receiving ascorbate and α-TP supplementation. The data in Fig. 2C are corrected for the release of ascorbate mentioned above.

Fig. 2D details the time course of the accumulation of

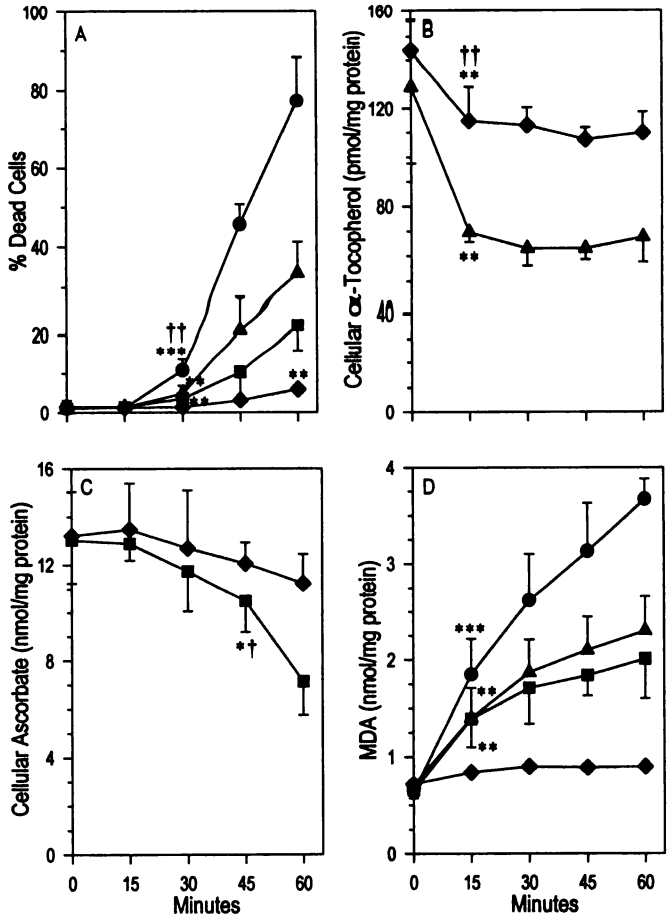

Fig. 2. Effect of 500 μM TBHP on cell killing (A), cellular vitamin E concentrations (B), cellular vitamin C concentrations (C), and accumulation of MDA (D) in the medium of hepatocytes that were cultured overnight without α-TP and ascorbate (●), with either α-TP (▲) or ascorbate (■) alone, or with both vitamins (♦). Hepatocytes were prepared and incubated overnight with or without 1.2 μM α-TP and/or 100 μM ascorbate. After 16–18 hr, cells were washed and placed in Williams' E medium without serum and additional α-TP and/or ascorbate. The cells were then incubated with 500 μM TBHP for 60 min. C, Vitamin C concentrations were also measured in a group of cells supplemented overnight with ascorbate alone and not exposed to TBHP. These measurements were taken at 0, 30, and 60 min. The rate of release of vitamin C from these cells was 3.48 nmol/mg of protein/hr (r^2 = 0.91). The cellular ascorbate values presented have been corrected for this nonspecific release of ascorbate. D, Hepatocytes supplemented with either vitamin alone were different from cells receiving both or no vitamins but were not different from each other ($p \leq 0.01$, Student-Neuman-Keuls). *Asterisks* and *daggers*, first points at which statistical differences are present. Values are mean ± standard deviation for three experiments. *, $p < 0.05$ compared with respective time zero. **, $p < 0.01$ compared with respective time zero. ***, $p < 0.001$ compared with respective time zero. †, $p < 0.05$ compared with other supplementation groups at the same time. ††, $p < 0.005$ compared with other supplementation groups at the same time.

MDA. In parallel with the cell killing, cultures that were not supplemented with either vitamin displayed the greatest rate and extent of lipid peroxidation, with the accumulation of MDA being significantly greater than in all other groups at each time point. Cells supplemented with either vitamin alone showed an intermediate rate and extent of the accumulation of MDA. There was no significant difference in the rate or extent of MDA accumulation when hepatocytes were supplemented with either vitamin alone. At the same time, cells supplemented with either vitamin alone had a significantly greater accumulation of MDA than did cells supplemented with both vitamins ($p < 0.01$). The latter cells had no significant evidence of lipid peroxidation.

Discussion

The current study documents the antioxidant function of the physiological content of vitamin C and argues that this vitamin does not seem to regenerate vitamin E in intact cells. Hepatocytes were isolated and cultured from ODS rats. Supplementation with ascorbate was necessary to maintain a physiological content of vitamin C in the hepatocytes. The vitamin C content remained ~12% of the wild-type in the absence of supplementation. The small residual activity of L-gulono-γ-lactone oxidase in ODS hepatocytes (37 pmol of ascorbate/min/mg of microsomal protein) presumably accounts for the fact that the ascorbate content was not zero (41, 42). The small amount of detectable ascorbate is not likely due to the Ester C in the drinking water because the livers were perfused and there was no change in the vitamin C content during overnight incubation (Table 1). In addition, the cultured cells supplemented with ascorbate release the vitamin into the medium (data not shown). Also, this small amount of ascorbate does not come from Williams' E medium (11 μM) because there is no detectable ascorbate in the medium, most likely due to its auto-oxidation (4). The 10% fetal calf serum added to the medium contributes no detectable ascorbate (data not shown).

On exposure to an oxidative stress, ODS hepatocytes were more sensitive to lethal injury than were comparable cells supplemented with ascorbate to maintain a physiological content of this vitamin. An increased formation of MDA preceded the increased killing of hepatocytes that were depleted of vitamin C (compare Fig. 2, D versus C). This result indicates that the antioxidant function of physiological levels of vitamin C relates, at least in part, to protection against peroxidative cell injury and is independent of the content of vitamin E. Also, supplemental ascorbate was an effective antioxidant in hepatocytes in the presence or absence of a physiological content of α-tocopherol. Thus, the ability of vitamin C to protect liver cells from oxidative cell killing did not depend on the vitamin E content of the cells.

Animal cells cannot synthesize vitamin E. Nevertheless, hepatocytes continue to secrete their stores of vitamin E on placement in cell culture (21, 43) and lose almost 90% of their cellular α-tocopherol content within 16–18 hr (4, 5, 21; Table 1). The 18 nM α-TP in Williams' E medium was not sufficient to maintain the cellular vitamin E content (21). Thus, by appropriate supplementation of the culture medium, the cellular content of either vitamin C or E could be independently manipulated, a situation that allowed analysis of the independent and dependent antioxidant functions of these vitamins.

The data presented in Figs. 1 and 2 document that the physiological content of either vitamin C or E alone (i.e., either vitamin C in the absence of vitamin E or vitamin E in the absence of vitamin C) has demonstrable antioxidant activity in a living system. The antioxidant protection afforded by either vitamin alone was intermediate between that seen in the absence and that seen in the presence of both vitamins. Thus, the physiological content of these two natural antioxidants seems to contribute independently to the overall antioxidant status of the cultured hepatocytes.

The current study also considers the interaction between vitamins C and E in the antioxidant defense of a living cell. In a variety of chemical or biochemical (i.e., nonliving) systems, vitamin C can reduce oxidized vitamin E (1, 2, 10–17). However, the data on the interaction between vitamin E and C in living systems have yielded conflicting results (4–8, 18, 44). In recent studies of the metabolism of vitamins E and C in cultured hepatocytes exposed to an oxidative stress, we found no evidence to support that vitamin C acts as a reductant of the vitamin E radical (4, 5). The decline in vitamin E occurred before the decline in vitamin C, a result that argues against vitamin C maintaining the cellular vitamin E content. However, the constitutive production of ascorbate by those cells complicated the interpretation of those studies.

In the current study, ODS hepatocytes were used because of their greatly deficient ascorbate content, thus permitting the analysis of only cellular vitamin E. As seen previously with wild-type cells, vitamin E levels declined before any loss of vitamin C in ODS hepatocytes containing physiological amounts of both vitamins E and C (compare Fig. 2, B versus C). In ODS hepatocytes depleted of vitamin C, the decline of vitamin E was greater than the decline of vitamin E in the presence of physiological levels of vitamin C (Fig. 2B). However, this result does not necessarily imply that vitamin C maintains the content of reduced vitamin E. The greater loss of vitamin E in the presence of a depletion of vitamin C is readily attributable to the increased peroxidation of lipids (Fig. 2D). In the absence of vitamin C, the more abundant products of lipid peroxidation readily account for the increased consumption of vitamin E. By directly scavenging the radicals derived from the metabolism of TBHP, vitamin C prevents the peroxidation of lipids and thus spares the depletion of vitamin E. The metabolism of TBHP has been shown to produce alkyl, alkoxyl, and peroxyl radicals in culture (45), and ascorbate has been shown to scavenge these oxygen radicals (1, 2, 9, 10, 17, 19, 20).

In summary, the data in the current study define the separate and comparable roles that the physiological contents of vitamins C and E play in the defense against oxidative cell injury in a living system. Vitamin C is a water-soluble antioxidant that presumably functions in the aqueous phase of the cell. In contrast, vitamin E is a lipid-soluble antioxidant that functions to scavenge radicals generated within the hydrophobic environment of cellular membranes. Both vitamins are necessary to fully defend a cell from oxidative attack because the depletion of either one alone increased the susceptibility to such injury. The relative ease with which cultured ODS hepatocytes were rendered deficient in vitamin C, vitamin E, or both allowed the respec-

tive and independent functions of these endogenous antioxidants to be readily determined.

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Send reprint requests to: John L. Farber, M.D., Department of Pathology, Thomas Jefferson University, 1020 Locust Street, Room 251, Philadelphia, PA 19107. E-mail: farber1@jeflin.tju.edu