Environmental and Molecular Mutagenesis 30:339–345 (1997)

Antimutagenic and Promutagenic Activity of Ascorbic Acid During Oxidative Stress

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Ascorbic acid (AA) has both antioxidant and prooxidant activities. However, there have not been any studies to elucidate the molecular mechanisms that determine whether AA functions as an anti- or a prooxidant during oxidative stress. The results of this study, using the Chinese hamster ovary cell line AS52 as a model system, demonstrate that there is a temporal relationship between the anti- and prooxidant activities of a physiologically relevant concentration of AA (50 µM) and oxidative stress. Treatment of cells with AA (50 µM) 24 hr prior to treatment of the cells with a radical generating system (RGS) results in a statistically significant inhibition of the cytotoxicity and mutagenicity associated with exposure of AS52 cells to oxidative stress. Conversely, cotreatment of cells with AA and the RGS results in a statistically significant increase in both the cytotoxic and mutagenic effects of oxidative stress when compared to cell populations exposed only to the RGS. The results, using a novel histochemical-computer image analysis system to detect hydrogen peroxide (H_2O_2), also demonstrate that there is a direct correlation between the ability of AA to decrease the levels of H_2O_2 in cells and the cytotoxic and mutagenic effects of oxidative stress. This study suggests that the time at which AA is administered in relation to exposure to oxidative stress has an impact on AA antimutagenic activity, and this may explain the conflicting results concerning the effectiveness of AA as a cancer chemopreventive agent. Environ. Mol. Mutagen. 30:339–345, 1997. © 1997 Wiley-Liss, Inc.

Key words: AS52 cells; ascorbic acid; oxidative stress; image analysis; hydrogen peroxide

INTRODUCTION

There is considerable interest in determining whether dietary compounds can be used successfully for cancer chemoprevention [Morse and Stoner, 1993]. One compound of interest is ascorbic acid (AA) [Lupulescu, 1994]. However, conflicting results have been obtained concerning the effectiveness of AA as a cancer chemoprotectant. Retrospective studies in humans have failed to demonstrate a correlation between the dietary use of AA and a decrease in the incidence of cancer [Jacob and Burri, 1996]. Also, there is little evidence supporting the hypothesis that high doses of AA can result in a greater degree of protection [McKeown-Eyssen et al., 1988]. However, these studies had limited population sizes and valid statistical conclusions could not be made. Therefore, AA effectiveness in cancer chemoprevention is still questionable.

AA is a well-characterized, water-soluble vitamin that possesses a variety of physiological properties. In addition to its antioxidant properties [Frei et al., 1989], AA is also capable of acting in a prooxidant manner [Stich et al., 1976]. The prooxidant activity of AA has been demonstrated in several studies by showing that high doses of AA induce sister chromatid exchange in vitro [Speit et al., 1980] and that the mutagenic activity of AA is enhanced by transition metals [Stich et al., 1976, 1979]. Thus, the ineffectiveness of AA as cancer chemoprotectant may be the result of its prooxidant activity. However, there have not been any studies to elucidate the biochemical/molecular mechanisms functioning at the cellular level that determine the relationship between the anti- and prooxidant activities of AA during oxidative stress.

The purpose of this study was to examine the relationship between the anti- and prooxidant activities of AA during oxidative stress by using the Chinese hamster ovary cell line AS52. AS52 cells lack the normal X-linked mammalian hypoxanthine-guanine-phosphoribosyl-transferase (*hprt*) gene, but have a single functional copy of the bacterial equivalent xanthine-guanine-phosphoribosyl-transferase (*gpt*) gene stably integrated in the genome [Tindall et al., 1987]. The results of this study demonstrate that there is a temporal relationship between the antioxi-

Contract grant sponsor: Ohio State University Comprehensive Cancer Center; Contract grant number: NIH NCI P30CA-16058-20; Contract grant sponsor: Ohio Cancer Research Associates.

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Received 15 November 1996; revised and accepted 17 May 1997.

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TABLE I. Effect of AA Pretreatment on AS52 Cell Survival and Mutation Frequency During Oxidative Stress[†]

Treatment	Survival[a] (%)	TG^r mutants/ 10^6 clonable cells[b]
Nontreated[c]	100.0 ± 24.6	17.4 ± 2.2
RGS	43.3 ± 17.6	54.7 ± 28.1*
10 μM AA + RGS	52.7 ± 19.7	73.0 ± 71.2
50 μM AA + RGS	101.0 ± 22.0	20.8 ± 2.7**
100 μM AA + RGS	77.8 ± 13.14	12.6 ± 8.9**

[†]AS52 cells (10^6) were treated with the RGS alone for 20 min as described in Materials and Methods, and this represents the positive control for these studies. Nontreated cells (10^6) represent the negative controls. Cells (10^6) were treated with the indicated concentration of AA for 24 hrs, washed, and treated with the RGS.

[a]Survival was determined 24 hrs posttreatment with the RGS. It was based upon the relative cloning efficiency of the treated cells when compared to nontreated controls. The absolute cloning efficiency of nontreated controls ranged from 78%–100%

[b]Mutation analyses were performed as described in Materials and Methods.

[c]Values represent the mean ± the standard deviation of a minimum of three experiments (fifteen samples). For statistical evaluations, the positive control was compared to the negative control ($*P < .05$), and the AA-treated populations were compared to the positive control ($**P < .05$).

dant and prooxidant activities of AA and oxidative stress. Furthermore, using a novel method for the detection and quantitation of H_2O_2, the data demonstrate that the antioxidative activity of AA is directly related to its ability to reduce H_2O_2 formation in AS52 cells during oxidative stress.

MATERIALS AND METHODS

Cell Culture

AS52 cells were obtained from Dr. Kenneth Tindall (National Institute of Environmental Health Sciences, Research Triangle Park, NC). AS52 cells were cultured in Ham's F-12 medium with L-glutamine (Sigma Chemical Co., St. Louis, MO) supplemented with 5% (v/v) dialyzed fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY) and MPA additives (10 mg/ml mycophenolic acid (MPA), 25 μg/ml adenine, 50 μM thymidine, 250 μg/ml xanthine, and 3 μM aminopterin) (Sigma Chemical Co.) at 37°C in a 5% CO_2 atmosphere.

Mutagenesis Studies

Mutation analysis was performed by using a modification of previously described procedures [Stankowski et al., 1986; Tindall et al., 1986, 1987; Tindall and Stankowski, 1989; Ariza and Williams, 1996]. Briefly, 48 hr prior to exposure to oxidative stress, cells (10^6) were cultured in F-12 medium containing FCS, but lacking MPA additives. On day 0, cells were washed twice with Hanks Balanced Salt Solution (HBSS) and exposed to oxidative stress. Oxidative stress consisted of exposing the cells to a radical generating system (RGS; xanthine oxidase (0.01 U/ml) and hypoxanthine (6.8 mg/ml) dissolved in F-12 medium lacking FCS) for 20 min at 37°C in 5% CO_2. After treatment, the cells were washed three times with HBSS, F-12 medium containing 5% FCS was added, and the cells were incubated at 37°C. On day +1, cells (10^6) were subcultured for mutagenesis studies and for cytotoxicity studies as described below. Cells treated with the RGS served as positive controls for the AA studies, while cells treated only with F-12 medium served as negative controls.

For the AA studies, cells (10^6) were either treated with AA 24 hours prior to exposure to the RGS or were cotreated with AA and the RGS. Cells treated with AA prior to exposure to the RGS were washed three times before treatment with the RGS. Following treatment with the RGS, the cells were washed three times with HBSS, F-12 medium containing 5% FCS was added, and the cells were incubated at 37°C. On day +1, cells (10^6) were subcultured for mutagenesis studies and for cytotoxicity studies, as described below.

For the mutagenesis studies, cells were subcultured on days +1 and +4, at a density of 10^6 cells per 100-mm dish. Selection was done on day +6, by culturing cells (10^6) in F-12 medium containing 5% FCS and 10 μM 6-thioguanine (TG) at a density of 2×10^5 cells per 100-mm dish. Cells were incubated for 7 days and examined for the development of TG-resistant (TG^r) clones. Only colonies containing 50 or more cells were counted. Relative cloning efficiency was determined simultaneously with the selection of TG^r mutants (described below).

Our historical spontaneous mutation frequency for nontreated AS52 cells in F-12 medium is 6.87 ± 4.81 TG^r mutants per 10^6 clonable cells [Ariza and Williams, 1996] (Aziza and Williams, unpublished results), which is a lower number than that previously reported for AS52 cells (15–30 × 10^6) [Stankowski et al., 1986; Tindall et al., 1986, 1987; Tindall and Stankowski, 1989]. We believe that these differences in spontaneous mutation frequencies reflect differences in the composition of the media and/or sera used in these studies.

Cytotoxicity and Cloning Efficiency of TG^r Clones

Cytotoxicity studies were performed on day +1 following treatment with AA and/or RGS. The cloning efficiency of the TG^r clones was determined on day +6. Briefly, cells (600) were plated at a density of 200 cells per 60-mm dish in F-12 medium containing 5% FCS and incubated at 37°C for 7 days. Cells were fixed with methanol:acetic acid:water (50:7:43) and stained with a 1% solution of crystal violet. Only colonies containing 50 cells or more were counted. The cytotoxicity of the various treatments was determined by comparing the cloning efficiency of cells exposed to AA and/or the RGS to the cloning efficiency observed for nontreated controls. In these studies, the absolute cloning efficiency for AS52 cells ranged from 78% to 100%.

In Situ Detection of H_2O_2

Cells were stained for H_2O_2 using a modification of the procedure described by Dannenberg et al. [1994]. Briefly, cells were cultured in either 100-mm plates or on chamber slides. Positive controls consisted of cells treated with the RGS. Negative controls consisted of nontreated cells and cells cotreated with the RGS and catalase (100 units). For determining the temporal effect of AA on in situ H_2O_2 formation during oxidative stress, cells were either treated with AA (50 μM) prior to treatment with the RGS, or cotreated with RGS and AA (50 μM). Immediately following treatment, cells were washed three times with HBSS and fixed with 4% paraformaldehyde for 4 hr in the dark at 4°C. Following fixation, the cells, either in suspension or growing as monolayers, were washed three times with 0.1 M Tris-HCl buffer, pH 7.0 and incubated in 20% sucrose overnight at 4°C in the dark. Cells were washed with 0.1 M Tris-HCl and incubated with 3,3′-diaminobenzidine (DAB) (1 mg/ml) and glucose (1 mg/ml) in 0.1 M Tris-HCl for 4 hours at 37°C. Cell suspensions were cytocentrifuged onto slides. Coverslips were placed on the slides using an aqueous mountant (Crystal Mount). Slides were viewed under a brightfield microscope (Zeiss Axio-

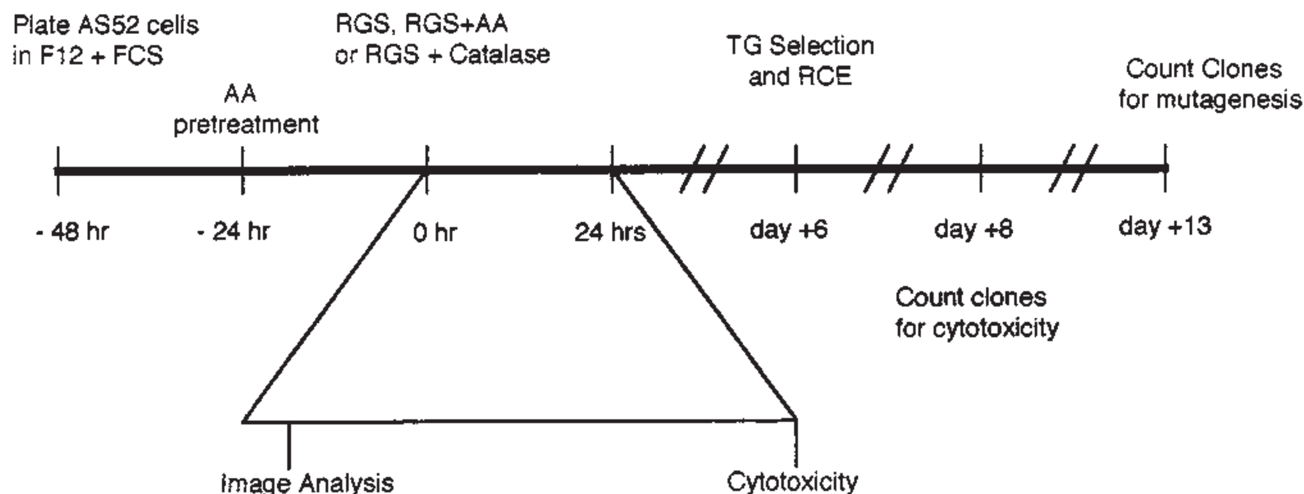

Fig. 1. Flow diagram for treatments and assays.

phot) at 400×. Video images were captured by a 768 × 494 pixel Optronics DEI 470 high-resolution video camera (Optronics Engineering). The camera is interfaced to a computer integrated with a frame grabber board containing the Roche Pathology Workstation software (Roche, Elon College, NC). The software enables the quantitation of staining intensity (optical density) in gray scale values, ranging from 0 (white) to 255 (black), and cell size (in pixels). The amount of staining in each cell was quantitated as the integrated optical density (IOD), which is calculated as cell size in pixels × its optical density (OD). Random fields were chosen, and a minimum of 200 cells were quantitated per slide. For statistical analyses, the IOD values of 50 randomly chosen cells per slide were used.

Statistical Analysis

Statistical analysis was performed by using paired Student's t test.

RESULTS

Our preliminary studies demonstrated that the treatment of AS52 cells with the RGS resulted in a statistically significant increase in cytotoxicity and mutation frequency of the *gpt* gene, when compared to nontreated controls (Table I). However, the cytotoxic and mutagenic effects of oxidative stress were prevented by the treatment of cells with AA prior to exposure to the RGS, but only at concentrations of AA >50 μM (Table I). Since the physiological concentration of AA is 50–60 μM [Frei et al., 1989], the 50-μM concentration of AA was employed in further studies.

To determine the temporal affect of AA on the cytotoxic and mutagenic effects associated with treatment of AS52 cells with the RGS, cells were treated either prior to exposure to the RGS, or they were cotreated with the RGS and AA. A flow diagram for these studies is shown in Figure 1. As shown in Table II, treatment of AS52 cells with AA 24 hrs prior to exposure to RGS resulted in enhanced cell survival and a statistically significant ($P < .005$) decrease in the mutation frequency of the *gpt* gene when compared to cells treated only with the RGS. Conversely, cotreatment of AS52 cells with AA (50 μM) and the RGS resulted in a statistically significant increase in the cytotoxicity ($P < .01$) and mutation frequency of the *gpt* gene ($P < .005$) in this cell population when compared to cells treated with the RGS alone. Treatment of cells with AA either 48 hr prior to treatment with the RGS or 24 hours after treatment with the RGS had no effect on cell viability or mutation frequency when compared to cells treated with the RGS (data not shown).

To determine the effect of AA on H_2O_2 formation in AS52 cells following treatment with the RGS, we used a novel technique that coupled a histochemical method to computer image analysis. DAB is a colorless, lipid-soluble compound that, in the presence of cellular peroxidases or catalase, or both, functions as a hydrogen donor for the degradation of H_2O_2. Upon oxidation, DAB polymerizes to form a brown insoluble phenazine polymer that precipitates in the cell [Litwin, 1979; Karnovsky, 1994]. Thus, the intensity of staining in a cell is dependent on cell size and also on the presence of cellular peroxidases and H_2O_2. By using the appropriate software, staining intensity (optical density) is quantitated in gray scale values ranging from 0 (white) to 255 (black), and cell size is quantitated in pixels. By using this method, it is possible to quantitate the relative amount of H_2O_2 present in a cell in any cell population as the integrated optical density (IOD), which is calculated as cell size in pixels × its optical density (OD).

As shown in Figure 2a, there is a low level of staining present in nontreated cells (negative controls); this is due to the production of H_2O_2 during normal cellular processes. However, as demonstrated by the histogram data in Figure 3A, the relative amount of H_2O_2 (IOD) that is

TABLE II. Temporal Effect of AA Treatment on AS52 Cell Survival and Mutation Frequency During Oxidative Stress[†]

Treatment	Survival[a] (%)	TG[r] mutants/ 10^6 clonable cells[b]
Nontreated[c]	100.0 ± 15.2	6.0 ± 1.9
RGS	60.0 ± 8.2	13.6 ± 3.6*
AA (24 hrs) + RGS	69.5 ± 20.7	3.1 ± 0.3**
AA + RGS	41.5 ± 11.4	21.3 ± 2.0**

[†]AS52 cells (10^6) were treated with the RGS alone for 20 min as described in Materials and Methods, and this represents the positive control for these studies. Nontreated cells (10^6) represent the negative controls. Cells (10^6) were either treated 50 μM AA 24 hrs prior to treatment with RGS or they were cotreated with 50 μM AA and RGS.
[a]Survival was determined 24 hrs posttreatment with the RGS. It was based on the relative cloning efficiency of the treated cells when compared to nontreated controls. The absolute cloning efficiency of nontreated controls ranged from 82%–100%.
[b]Mutation analyses were performed as described in Materials and Methods.
[c]Values represent the mean ± the standard deviation of a minimum of three experiments (fifteen samples). For statistical evaluations, the positive control was compared to the negative control (*$P < .05$), and the AA-treated populations were compared to the positive control (**$P < .005$).

formed in a cell varies greatly among other cells within the population. Treatment of AS52 cells with the RGS resulted in increased staining of the cells (Fig. 2b) when compared to the nontreated controls (Fig. 2a). This is also shown in the histogram profiles; there is a shift in the profile to the right in RGS-treated cells (Fig. 3b) when compared to the profile of nontreated controls (Fig. 3a), demonstrating that cells treated with the RGS contain higher amounts of H_2O_2 per cell than cells in the nontreated population. To demonstrate that the increased staining in AS52 cells treated with RGS was due to H_2O_2, cells were cotreated with RGS and catalase. The inclusion of catalase prevented the staining of cells treated with RGS (Fig. 2d) and caused a shift in the histogram profile to the left (Fig. 3d) when compared to cells treated only with RGS (Figs. 2b and 3B, respectively).

To determine the effect of AA (50 μM) on H_2O_2 formation in AS52 cells treated with the RGS, cells were either treated with AA 24 hrs prior to treatment with the RGS or cotreated with AA and RGS. Treatment of cells with AA prior to treatment with the RGS completely inhibited staining of cells with DAB (Fig. 2c) when compared to cells treated with RGS (Fig. 2b; positive controls), and the staining intensity in these cells more closely resembled the staining intensity observed in nontreated cells (Fig. 2a; negative controls). This is also reflected in the histogram profile, which demonstrated that treatment of cells with AA prior to treatment with the RGS (Fig. 3c) resulted in decreased amounts of H_2O_2 in these cells when compared to cells treated only with the RGS (Fig. 3b). The histogram profile of cells treated with AA before treatment with RGS (Fig. 3c) closely resembled the profile that was observed in nontreated cells (Fig. 3a). Conversely, cells cotreated with AA and the RGS exhibited the same degree of staining and a similar histogram profile as cells treated only with the RGS (data not shown).

Statistical analyses using the mean IOD value of 50 randomly selected cells from each treatment population demonstrated that there was a significant increase in the staining of cells treated with RGS (positive control) when compared to nontreated cells ($P < .025$) or cells cotreated with catalase and RGS ($P < .005$) (Table III). The treatment of cells with AA (50 μM) prior to the treatment of the cells with RGS resulted in a significant ($P < .005$) decrease in the staining of the cells when compared to the staining that was observed in cells treated with only the RGS. The level of staining observed in cells treated with AA prior to RGS was not statistically different from the staining levels that occurred in nontreated cells or in cells cotreated with catalase and RGS. Conversely, there was no significant difference in the amount of staining observed in cells cotreated with AA and the RGS when compared to cells treated with only the RGS. However, there was a significant increase ($P < .025$) in the amount of staining in cells cotreated with AA and RGS when compared to nontreated controls, cells cotreated with catalase and RGS, and cells treated with AA prior to treatment with RGS.

DISCUSSION

Reactive oxygen intermediates (ROI), such as superoxide anion, H_2O_2, and the hydroxyl radical, have been implicated in several degenerative processes, including carcinogenesis [Halliwell and Gutterridge, 1990; Ames and Shigenaga, 1993]. Such studies have stimulated much interest in identifying antioxidative compounds present in the diet that can be used for cancer chemoprevention. One such dietary component is AA, which has a major role in protecting cells against damage from oxidative stress. Not only does AA possess antioxidative properties, but it also contributes to the antioxidative status of a cell by reducing both vitamin E, a major membrane-associated antioxidant [Burton, 1994], and/or glutathione, the major cellular antioxidant [Meister, 1994]. However, the antioxidative properties of AA are concentration-dependent [Wayner et al., 1986], and, under certain conditions, it may act as a prooxidant and induce DNA damage [Galloway and Painter, 1979; Stich et al., 1979; Speit et al., 1980] and mutagenesis [Stich et al., 1976]. At the present time, however, the biochemical/molecular mechanisms that determine whether AA will function as an antioxidant or a prooxidant are unknown.

The results of this study demonstrate that there is a temporal relationship between the anti- and prooxidant

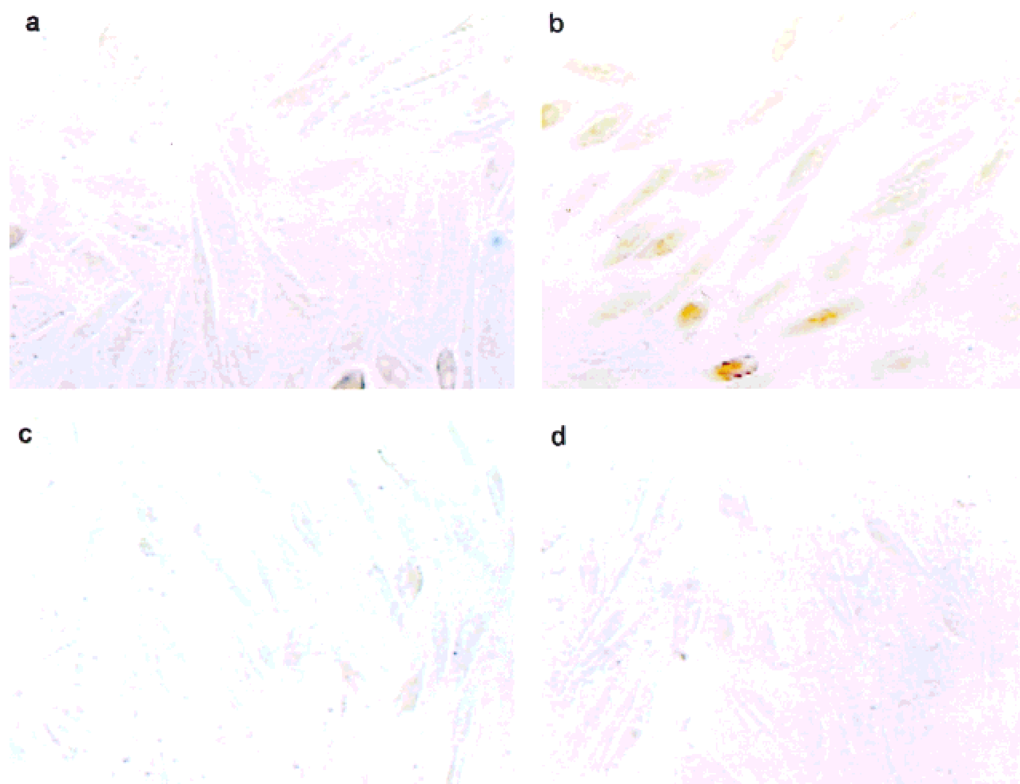

Fig. 2. In situ demonstration of H_2O_2 formation using DAB histochemical-image analysis. Cells were treated and examined for H_2O_2 formation as described in Materials and Methods. 400×. **a:** Nontreated cells. **b:** Cells treated with the RGS. **c:** Cells treated with AA (50 μM) for 24 hours before treatment with the RGS. **d:** Cells cotreated with the RGS and catalase.

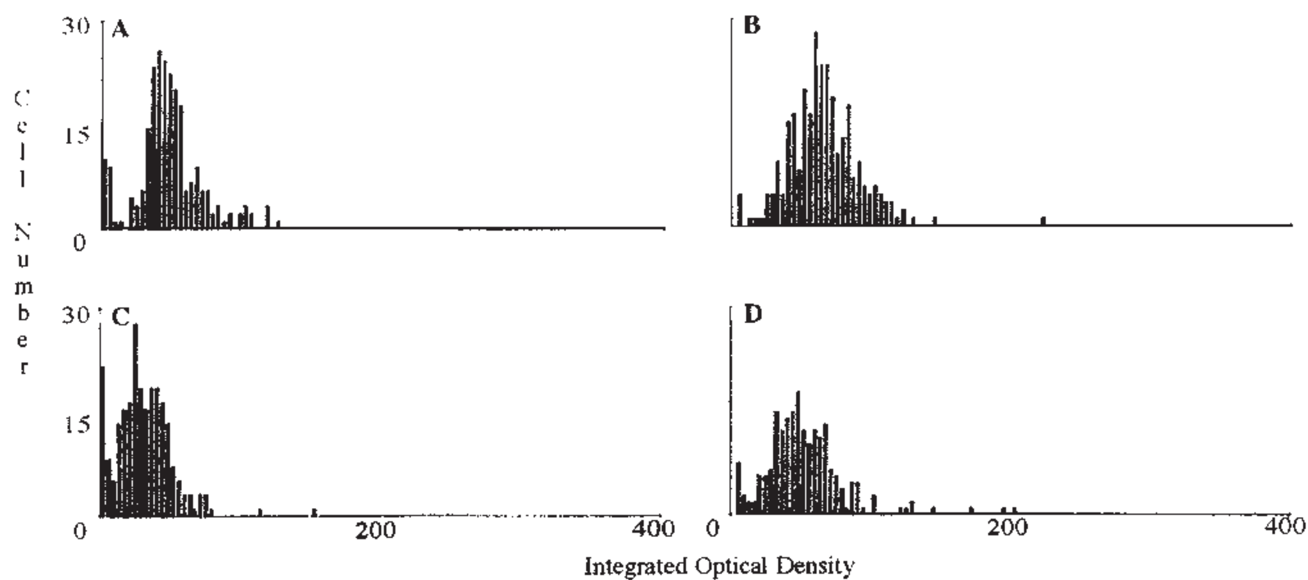

Fig. 3. Histogram profiles demonstrating H_2O_2 formation in AS52 cells. Cells were treated and examined for H_2O_2 formation as described in Materials and Methods. **A:** Nontreated cells. **B:** Cells treated with the RGS. **C:** Cells treated with AA (50 μM) for 24 hours prior to treatment with the RGS. **D:** Cells cotreated with the RGS and catalase.

TABLE III. Measurement of H_2O_2 In Situ by Computer Image Analysis[†]

Treatment	Mean IOD[a]
Nontreated	118.72 ± 52.41[b]
RGS	146.85 ± 74.81*
RGS + catalase	107.90 ± 54.55**
RGS + AA pretreatment	94.32 ± 47.76**
RGS + AA cotreatment	144.27 ± 78.50

[†]Computer image analysis was performed as described in Materials and Methods.
[a]IOD, integrated optical density, was determined as described in Materials and Methods.
[b]Statistical analysis was performed on a minimum of 50 cells from each treatment population using paired Student's t test. *$P < .025$ when compared to nontreated controls **$P < .005$ when compared to RGS-treated cells.

activities of a physiologically relevant concentration of AA in cells undergoing oxidative stress. AA (50 μM) functions as an antioxidant, provided cells are treated with AA prior to treatment with the RGS. This is demonstrated by a decrease in both the cytotoxic and mutagenic effects associated with treatment of AS52 cells with the RGS. Conversely, cotreatment of AS52 cells with the same concentration of AA and the RGS results in an increase in the cytotoxic and mutagenic effects of oxidative stress when compared to cells treated with only the RGS, indicating that, under these conditions, AA functions as a prooxidant. Treatment of cells with AA for time periods >24 hrs prior to, or 24 hrs after, treatment with the RGS has no effect on the cytotoxic or mutagenic effects associated with exposure of cells to oxidative stress.

In our model, both superoxide anion and H_2O_2 are produced by the RGS. While the cytotoxicity resulting from exposure to the RGS could be due in part to the oxidation of membrane components by superoxide anion, it is more probable that the cytotoxic and mutagenic effects of the RGS are due to H_2O_2. While superoxide anion is highly reactive, H_2O_2 is relatively stable and diffuses into cells and nuclei. In the presence of transition metals, that are complexed to DNA or proteins, H_2O_2 can be transformed into highly reactive hydroxyl radicals that can oxidize both DNA and proteins [Halliwell and Gutterridge, 1990]. This premise is supported by the DAB studies. DAB does not react with superoxide anion [Litwin, 1979; Dannenberg et al., 1994; Karnovsky, 1994], and our results, using DAB image analysis, demonstrates a direct correlation between the formation of H_2O_2 in cells and the antioxidative activity of AA.

What mechanism(s) could be responsible for temporally regulating the anti- and prooxidative effects of AA? It may be the result of transport of AA. Recent studies have demonstrated that at least two distinct transport systems are involved with AA uptake in cells. In some cells, such as astrocytes, AA is actively transported by a Na^+-dependent mechanism [Siushanasian and Wilson, 1995]. In other cells, such as lymphocytes, the oxidized form of AA, dehydroascorbate (DHA), is taken into cells by the glucose-facilitated transport system, where DHA is reduced, presumably by a glutathione-mediated reaction [Vera et al., 1993; Welch et al., 1995]. Thus, if CHO cells only accumulate DHA, the treatment of AS52 cells with AA prior to treatment with the RGS would allow for the transport of DHA and its reduction to AA. This would allow for the development of a reduced environment in the cell due to the presence of AA and possibly due to the upregulation of glutathione synthesis [Meister, 1994]. Conversely, in cells cotreated with AA and the RGS, the cells would not have sufficient time to transport DHA and provide a reduced protective environment in the cells. Furthermore, under these conditions, AA could be oxidized by the RGS [Giblin et al., 1984] leading to the formation of ascorbate anion and/or DHA and could possibly cause cell death and/or the depletion of glutathione [Meister, 1994]. Any of these mechanisms could account for the increased cytotoxic and mutagenic effects observed with the cotreatment of AS52 cells with the RGS and AA.

While additional studies are required to elucidate the mechanism(s) of AA transport in AS52 cells, the results of this study demonstrate that there is a temporal relationship between the antioxidative/prooxidative properties of AA and damage induced by oxidative stress. Furthermore, these results demonstrate that the AS52 cell line can be used to elucidate the molecular factors involved with determining the relationship between oxidative stress and the anti- and prooxidative activities of AA.

ACKNOWLEDGMENTS

We thank Dr. Kenneth Tindall, Laboratory of Environmental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, for providing the AS52 cell line and also for his technical assistance.

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Accepted by—
E. Zeiger