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Ascorbic Acid-Dehydroascorbate Induces Cell Cycle Arrest at G_2/M DNA Damage Checkpoint During Oxidative Stress

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Reactive oxygen species induce cellular damage and have been implicated as mediators for cellular signaling pathways. However, a linkage between the cellular redox status and cell cycle progression has not been demonstrated. We previously demonstrated, using the Chinese hamster ovary cell line AS52, that the cytotoxic and mutagenic effects of oxidative stress is prevented by ascorbic acid (AA), but only when cells are treated with AA prior to treatment with the stressor. To elucidate the mechanism(s) responsible for this effect, we determined the effect of AA on cell cycle progression during oxidative stress. Flow cytometric analyses demonstrated that treatment of AS52 cells with AA (50 μM), prior to treatment with a radical generating system (RGS), enhanced cell cycle arrest at the G_2/M DNA damage checkpoint when compared to cells treated with RGS. AA had no effect on cell cycle progression in the absence of oxidative stress. Furthermore, under conditions that prevent the reduction of dehydroascorbate (DHA), the oxidized form of AA, cell cycle arrest was also induced at the G_2/M DNA damage checkpoint. These observations demonstrate that during periods of oxidative stress, AA functions as an antioxidant and DHA enhances transient arrest at the G_2/M checkpoint by delaying the activation of cyclin B-cdc2. These results suggest the presence of a unique redox mechanism for the regulation of cell cycle progression and also demonstrate a novel mechanism by which AA protects cells from damage due to oxidative stress. Environ. Mol. Mutagen. 33:144–152, 1999

Key words: AS52 cells; ascorbic acid; dehydroascorbate; cell cycle arrest

INTRODUCTION

Ascorbic acid (AA) is the major water soluble antioxidant present in cells and plasma [Frei et al., 1988, 1989]. Numerous in vitro and in vitro studies have demonstrated the antimutagenic [Frei et al., 1988, 1989; Fraga et al., 1991; Krinsky, 1993; Sweetman et al., 1997] and anticlastogenic effects of AA [Shamberger, 1984; Krishna et al., 1986]. Conversely, other studies have demonstrated that under certain conditions AA functions as a prooxidant and increases DNA damage [Stich et al., 1976, 1979; Galloway and Painter, 1979; Rosin et al., 1980; Speit et al., 1980]. Interpretation of data from such studies have been based on the free-radical scavenging and autooxidizing properties of AA [reviewed in Niki, 1991]. AA has other regulatory roles in several biological processes, including transcription and translation [Lyons and Schwarz, 1984; Leboy et al., 1989; Huang et al., 1993; Sullivan et al., 1994] and it has been suggested that dehydroascorbate (DHA), the oxidized form of AA, may have a role in regulating cell cycle progression [Edgar, 1970]. However, the role of AA and/or DHA in these processes has not been elucidated.

Progression through the cell cycle is regulated by complex protein–protein interactions between cyclins and cyclin-dependent protein kinases (cdks) [Morgan, 1995; Stillman, 1996; Elledge, 1996]. These cyclin-cdk complexes are positively and negatively regulated by phosphorylation and by the binding of cyclin kinase inhibitors (ckI) to the complex. Following DNA damage, cells can be transiently arrested at the G_1/S, S, or G_2/M DNA damage checkpoints, and the DNA is either repaired or the cell undergoes apoptosis. While it is well established that p53 has a pivotal role in inducing cell cycle arrest at the G_1/S DNA damage checkpoint, recent studies suggest that transient arrest at the

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G_2/M DNA damage checkpoint is a p53 independent process that involves the inactivation/sequestering of cdc25C [Furnari et al., 1997; Sanchez et al., 1997; Peng et al., 1997; Poon et al., 1997]. This prevents the dephosphorylation and activation of the mitosis-promoting factor, cyclin B-cdc2, which causes cells to arrest at the G_2/M DNA damage checkpoint. The reactive oxygen intermediate hydrogen peroxide (H_2O_2) induces a variety of responses in cells, including cell death, cellular senescence [Chen and Ames, 1994; Chen et al., 1995] and transient cell cycle arrest at either the G_1/S or G_2/M DNA damage checkpoint [Gelvan et al., 1995; Clopton and Saltman, 1995; Wiese et al., 1995; Wharton, 1995]. While arrest at the G_1/S DNA damage checkpoint probably involves p53, the mechanism by which H_2O_2 induces arrest at the G_2/M DNA damage checkpoint is unknown.

We previously demonstrated, using the Chinese hamster ovary cell line AS52 as a model system, that a physiologically relevant concentration of AA (50 μM) prevented the enhanced cytotoxicity and mutagenicity observed in AS52 cells treated with an oxidative stressor, but only when cells were treated with AA prior to treatment with the oxidative stressor [Bijur et al., 1997]. The results of the present study demonstrate that AA enhances transient cell cycle arrest at the G_2/M DNA damage checkpoint during oxidative stress. Furthermore, under conditions that prevented the reduction of DHA, the oxidized form of AA, cell cycle arrest was also induced during oxidative stress. These observations demonstrate that during periods of oxidative stress, AA functions as an antioxidant and DHA enhances transient arrest at the G_2/M checkpoint by delaying the activation of cyclin B-cdc2. These results suggest the presence of a unique redox mechanism for the regulation of cell cycle progression and also demonstrate a novel mechanism by which AA protects cells from damage due to oxidative stress.

MATERIALS AND METHODS

Materials

AA, propidium iodide, RNase A, sodium citrate, polyethylene glycol (PEG) 8000, buthionine sulfoximine (BSO), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). DHA was purchased from Aldrich Chemicals (Milwaukee, WI). Nitrocellulose, ECL horseradish peroxidase substrate, and chemiluminescence film (Hyperfilm) were purchased from Amersham Life Sciences (Arlington Heights, IL). Mouse monoclonal antibodies against cdc2 (clone 17), cyclin B1 (clone GSN1), goat anti-mouse horseradish peroxidase (HRP) conjugated IgG, and protein A/G conjugated agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against p53 (clone PAb240), cyclin A (clone E23), and normal mouse IgG were purchased from Oncogene Research Products (Cambridge, MA). Mouse monoclonal antibody against phosphotyrosine (clone PY20) was purchased from Transduction Laboratories (Lexington, KY). All chemicals and enzymes were of the highest purity available.

Cell Culture and Treatments

AS52 cells were obtained from Dr. Kenneth Tindall (National Institute of Environmental Health Sciences, Research Triangle Park, NC). AS52 cells were grown and maintained, as described previously, at 37°C in a humidified 5% CO_2 environment in Hams F-12 medium supplemented with 5% (v/v) dialyzed fetal calf serum (FCS) and MPA additives (10 μg/ml mycophenolic acid, 25 μg/ml adenine, 50 μM thymidine, 250 μg/ml xanthine and 3 μM aminopterin) [Ariza and Williams, 1996; Bijur et al., 1997].

AS52 cells were treated as described previously [Bijur et al., 1997]. Briefly, cells (10^6) were cultured 24 hr prior to the addition of the radical generating system (RGS, xanthine oxidase (0.01 unit/ml) and hypoxanthine (6.8 mg/ml) dissolved in F-12 medium lacking FCS) in Hams F-12 medium containing 5% FCS. Cells were washed with Hank's Balanced Salt Solution (HBSS), and at the indicated times either AA (50 μM) or DHA (50 μM) in F-12 medium containing 5% FCS was added. For studies with BSO, cells (10^6) were cultured in F-12 medium containing 5% FCS 48 hr prior to treatment with RGS and fresh medium containing BSO (0.01 M) was added 24 hr prior to treatment with the RGS. BSO was maintained in the medium during treatment with AA or DHA. Following AA or DHA treatment, cells were washed three times with HBSS and treated with RGS for 20 min at 37°C in 5% CO_2. After treatment with 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 for an additional 24 hr.

Cytotoxicity studies were performed as described previously [Bijur et al., 1997]. Briefly, cells (600) were plated at a density of 200 cells per 60 mm dish in F-12 medium containing 5% FCS and incubated 7 days at 37°C. 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 in the treatment group to the cloning efficiency of untreated controls. Absolute cloning efficiency of AS52 cells ranged from 82–100%.

Cell Cycle Analysis

Twenty-four hours following treatment with RGS, cells (10^6) were removed by trypsinization, washed three times with cold 4 mM sodium citrate stained with propidium iodide (in a solution containing 4 mM sodium citrate, 3% (w/v) PEG 8000, 100 μg/ml propidium iodide, 180 units/ml RNase A and 0.01% (v/v) Triton X-100 for 20 min at 37°C, followed by incubation in a solution containing 0.4 M NaCl, 3% PEG, 100 μg/ml propidium iodide and 0.01% Triton X-100 for 1 hr at 4°C). Following staining, cell cycle analysis was performed using a Coulter EPICS XL flow cytometer. For analysis, the primary G_1 peak of untreated cells was set on channel 200 of a 1024 channel histogram and the settings were not changed during the course of the analysis. Percentage of cells in G_0/G_1, S, and G_2/M were determined using Multicycle AV Version 3.0. Analysis was on a minimum of 3.0×10^4 events. For a positive control, cells were treated with nocodazole (4 μg/ml) for 24 hr. Additional controls consisted of untreated cells, cells treated with RGS for 20 min, and cells treated with either AA or DHA (50 μM) for 2 hr. A minimum of three analyses were performed.

Western Analyses and Immunoprecipitations

Twenty-four hours following treatment with RGS, cells were washed with cold 10 mM Tris-Cl, pH 7.0 containing 50 μM phenylmethylsulfonylfluoride, 3 units/ml aprotinin and 1 mM sodium orthovanadate (lysis buffer), harvested, resuspended in lysis buffer, and lysed by sonication (20 1-sec treatments at 40% duty cycle setting 5 for microtip probe using a Branson Model 350 sonicator). The homogenates were centrifuged at 14,000*g* for 5 min and the supernatants were stored at -70°C until use.

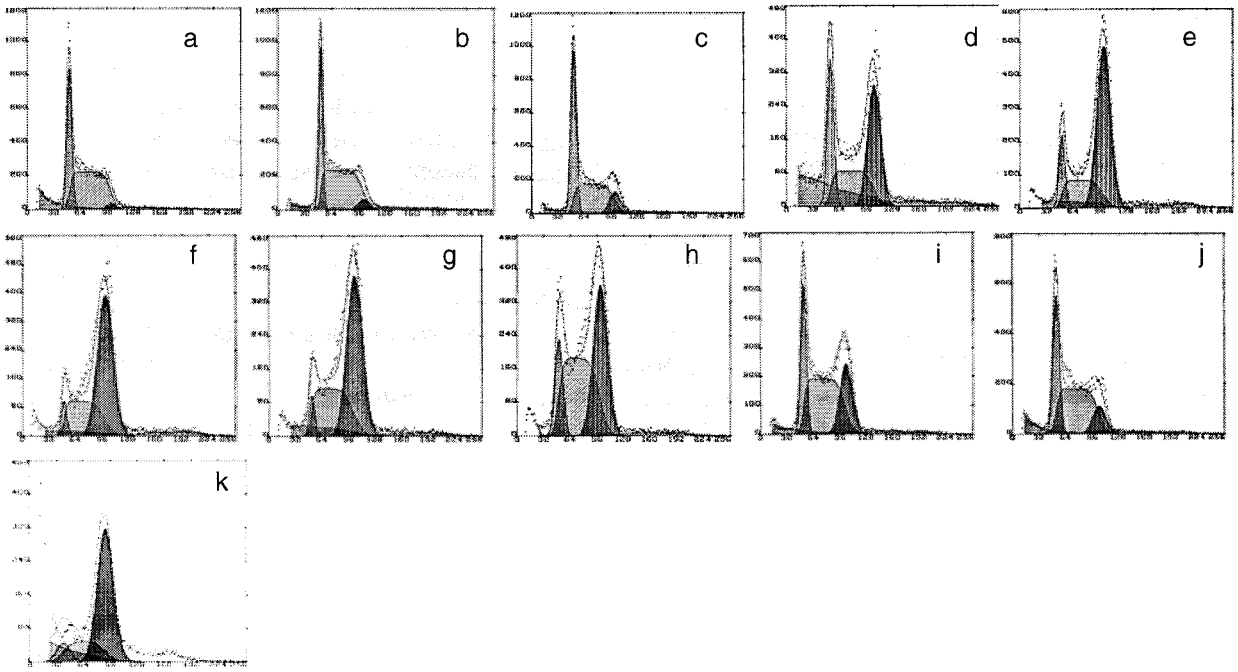

Fig. 1. Kinetic analysis of AA-induced cell cycle arrest. AS52 cells (10^6) were treated with 50 μM AA for the indicated times followed by treatment with RGS, stained with propidium iodide and examined by flow cytometry as described in Material and Methods. **a**: Untreated cells; **b**: RGS treatment; **c**: AA treatment; **d**: AA + RGS, 0 time; **e**: AA treatment 10 min prior to RGS; **f**: AA treatment 30 min prior to RGS; **g**: AA treatment 60 min prior to RGS; **h**: AA treatment 2 hr prior to RGS; **i**: AA treatment 4 hr prior to RGS; **j**: AA treatment 24 hr prior to RGS; **k**: nocodazole control.

Total protein in the supernatants was determined by Commassie blue staining (BioRad Protein Determination Kit, Richmond, CA) with bovine serum albumin as the standard. Proteins (20 μg) from the supernatants were separated on 10% SDS-PAGE and transferred by electroblotting to nitrocellulose using a standard transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol). Membranes were blocked with blotto (10 mM Tris-Cl pH 7.5 containing 0.05% (v/v) Tween-20 and 5% (w/v) nonfat dry milk) for 1 hr and immunoprobed using mouse monoclonal antibodies against p53, cdc2, cyclin A, or cyclin B1 (diluted in blotto) for 1 hr at 25°C. Normal mouse IgG served as a negative control. For the detection of phosphotyrosine, the membranes were blocked as described by the manufacturer of the anti-phosphotyrosine antibody with 10 mM Tris-HCl, pH 7.5, containing 0.5% (v/v) Tween-20 and 1% (w/v) BSA. Proteins and phosphotyrosine were detected by chemiluminescence using goat anti-mouse IgG conjugated to horseradish peroxidase and ECL HRP substrate. Bands were quantitated densitometrically using Sigma Scan.

For immunoprecipitations, protein lysates were prepared as described above. Protein (200 μg) was added to the immunoprecipitation buffer (10 mM PBS, pH 7.5, containing 50 μM phenylmethylsulfonylfluoride, 3 units/ml aprotinin, 1 mM sodium orthovanadate) and pretreated with normal mouse IgG (1 μg/ml) and protein A/G conjugated agarose beads (20 μl) for 1 hr at 4°C. Following centrifugation, the pellets were suspended in electrophoresis sample buffer (50 mM Tris-Cl, pH 6.5, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% (w/v) bromophenol blue) and boiled for 5 min. These samples were used as nonspecific controls. Anti-cyclin A or B antibody (1 μg/ml) and protein A/G conjugated agarose beads (20 μl) were added to the supernatants and the mixture was incubated with mixing for 24 hr at 4°C. Following incubation, samples were centrifuged and the pellets resuspended in electrophoresis sample buffer and processed as described above. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoprobed with the designated antibody as described above.

TABLE I. Effect of AA and RGS on Cell Cycle Progression*

	Percentage of cells		
Treatment	G_0/G_1	S	G_2/M
Untreated	35.7	61.4	3.0
RGS	42.7	45.5	11.8
AA	33.8	61.0	6.0
AA + RGS (0 time)	26.7	32.0	41.3
AA (10 min) + RGS	12.5	25.1	62.4
AA (30 min) + RGS	5.5	31.7	62.8
AA (60 min) + RGS	5.4	36.4	58.3
AA (2 hr) + RGS	13.6	46.6	39.9
AA (4 hr) + RGS	24.2	51.1	24.6
AA (24 hr) + RGS	31.9	54.5	13.5
Nocodazole	8.3	27.0	69.7

*AS52 cells were treated with 50 μM AA for the indicated times and then with RGS, stained with propidium iodide, and examined by flow cytometry as described in Materials and Methods. Controls cells were untreated, treated with RGS for 20 min, or treated with 50 μM AA for 2 hr.

RESULTS

Effects of AA on Cell Cycle Progression During Oxidative Stress

To determine whether AA was effecting cell cycle progression during periods of oxidative stress, asynchronously growing AS52 cells were treated with AA for various times prior to treatment with RGS and examined by flow cytom-

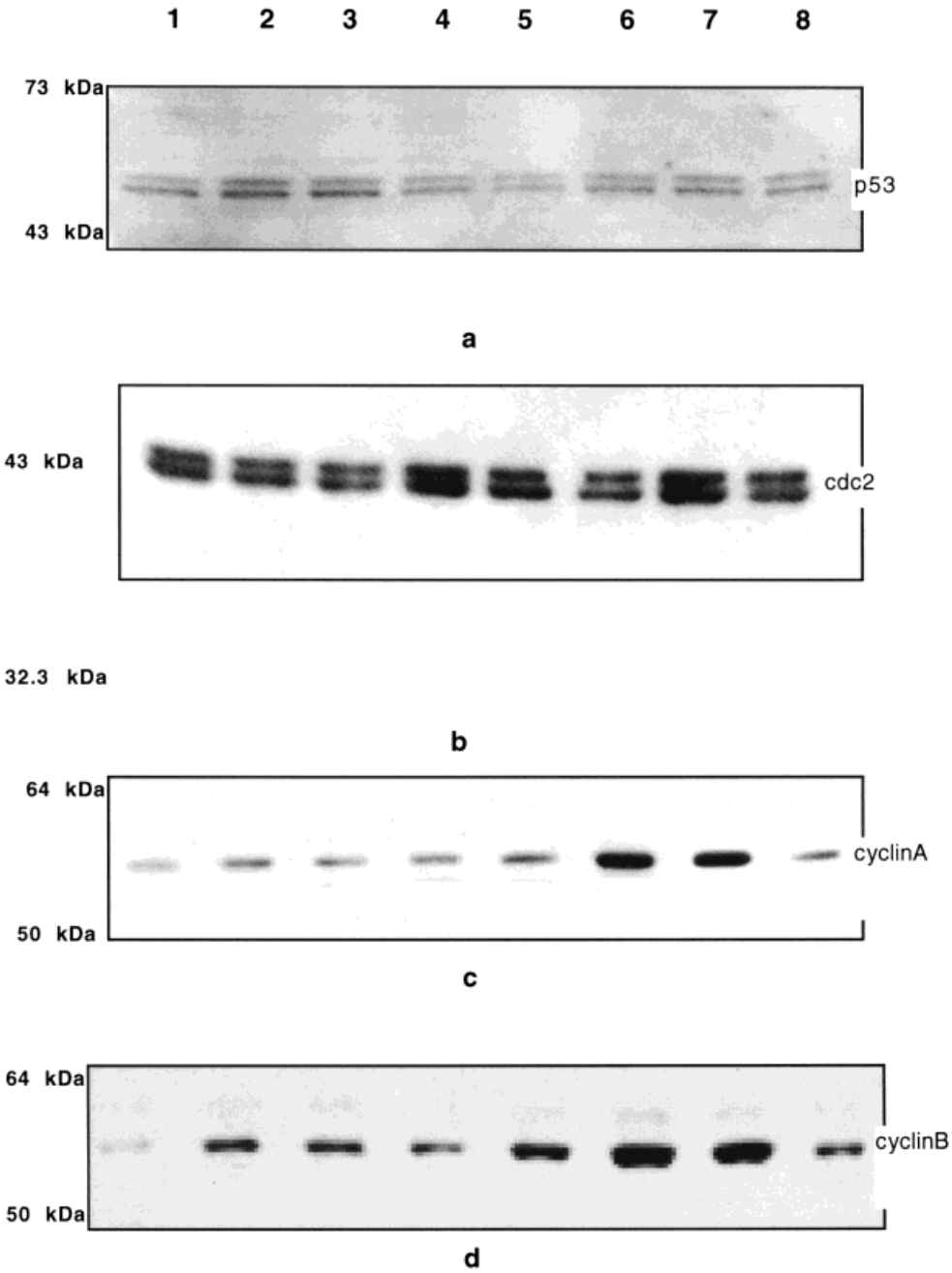

Fig. 2. Effect of AA and oxidative stress on cellular levels of p53, cdc2, cyclin A, and cyclin B. Cells were treated and protein extracts were analyzed by Western blotting as described in Material and Methods. Lane 1: untreated cells; Lane 2: RGS-treated cells; Lane 3: AA (50 μM) treated cells; Lane 4: AA- and RGS-treated cells, 0 time; Lane 5: cells treated with AA 30 min prior to RGS treatment; Lane 6: cells treated with AA 60 min prior to RGS treatment; Lane 7: cells treated with AA 2 hr prior to RGS treatment; Lane 8: cells treated with AA 24 hr prior to RGS treatment. **a**: p53; **b**: cdc2; **c**: cyclin A; **d**: cyclin B1.

etry (Fig. 1; Table I). Treatment of cells with RGS resulted in a slight increase in the percentage of cells in G_0/G1 and G_2/M (Fig. 1b) when compared to untreated controls (Fig. 1a). Conversely, AA (50 μM) had no effect on cell cycle progression (Fig. 1c). Higher concentrations of AA (0.1 and 1 mM) had no effect on the progression of AS52 cells through the cell cycle (data not shown). Treatment of cells with AA prior to treatment with RGS or co-treatment of cells with AA and RGS resulted in a significant increase in the percentage of cells arrested at G_2/M (Fig. 1d–j). Maximal G_2/M arrest occurred in cells treated with AA 10–60 min (Fig. 1e–g) prior to treatment with RGS and minimal arrest occurred in cells treated either 24 hr before (Fig. 1j) or after treatment (data not shown) with RGS.

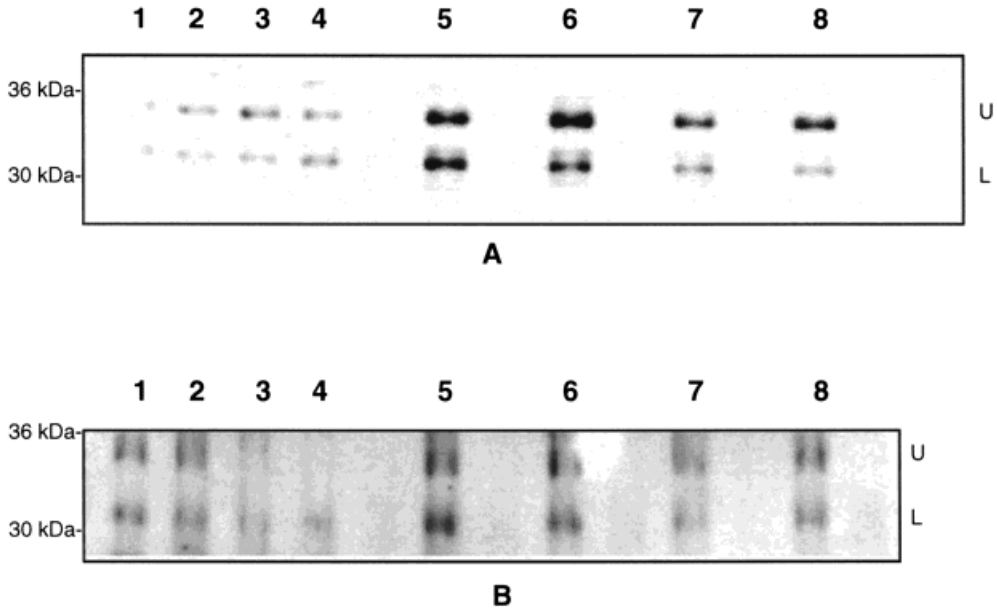

Fig. 3. Effect of AA and oxidative stress on cyclin B-cdc2. Protein lysates were prepared as described in Material and Methods. Extracts (200 μg) were precleared using normal mouse IgG and protein A/G conjugated agarose beads. Cyclin B-cdc2 complexes were immunoprecipitated as described in Material and Methods using mouse monoclonal anti-cyclin B1 and protein A/G conjugated agarose beads. SDS-PAGE and immunoblotting were performed as described in Material and Methods using mouse monoclonal antibodies against cdc2 or phosphotyrosine. Proteins were detected by chemiluminescence. Lanes: 1) untreated controls; 2) extracts from RGS-treated cells; 3) extracts from AA-treated cells; 4) extracts from cells co-treated with AA and RGS (0 time); 5) extracts from cells treated with AA 30 min prior to treatment with RGS; 6) extracts from cells treated with AA 1 hr prior to treatment with RGS; 7) extracts from cells treated with AA 2 hr prior to treatment with RGS; and 8) extracts from cells treated with AA 24 hr prior to treatment with RGS. **A**: Western blotting with anti-cdc2 antibody; U: T14-Y15-T161 form of cdc2; L: T14-T161 and Y15-T161 forms of cdc2. **B**: Western blotting with anti-phosphotyrosine antibody; U: T14-Y15-T161 form of cdc2; L: Y15-T161 form of cdc2.

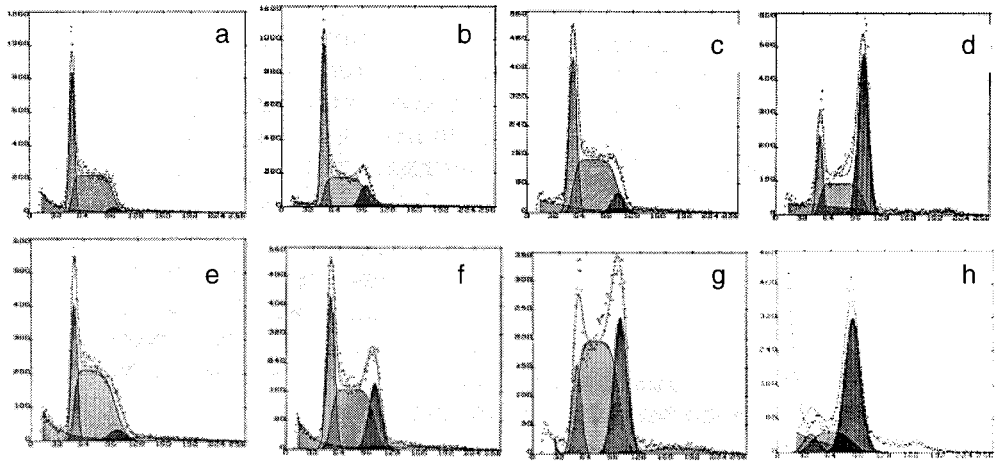

Fig. 4. Effect of DHA and BSO treatment on cell cycle progression. Cell treatments and cell cycle analysis were performed as described in Materials and Methods. **a**: Untreated cells; **b**: RGS treatment; **c**: DHA treatment; **d**: DHA treatment 2 hr, then RGS; **e**: BSO treatment; **f**: BSO and RGS treatment; **g**: BSO, DHA (2 hr) treatments, then RGS; **h**: nocodazole control.

Effects of AA on Expression of p53 Cyclins A and B and the Cyclin B-cdc2 Complex

To begin to elucidate the mechanism(s) by which AA enhances arrest at the G_2/M checkpoint during oxidative stress, we determined whether AA induced any changes in the levels of proteins involved in cell cycle progression or checkpoint control during oxidative stress. There were no changes in the levels of p53 or cdc2 in treated cells when compared to the controls (Fig. 2a,b, respectively). We believe that the multiple bands seen in SDS-PAGE gels from extracts of asynchronously growing cells (Fig. 2a,b) represent different phosphorylated forms of p53 and cdc2 that are

recognized by the monoclonal antibodies used in these studies. Conversely, there was an increase in both cyclin A and cyclin B levels in cells treated with AA prior to treatment with RGS (Fig. 2c,d). The maximum increase in cyclin A and cyclin B (17-and 6-fold, respectively) occurred in cells treated with AA for 60 min prior to treatment with RGS. Immunoprecipitation of cyclin B-cdc2 complexes with anti-cyclin B1 monoclonal antibody, demonstrated that there was an accumulation of at least two electrophoretically distinct forms of cdc2 bound to cyclin B1 in cells treated with AA and RGS (Fig. 3A). Immunoprobing with anti-phosphotyrosine antibody demonstrated that the forms of cdc2 complexed to cyclin B were phosphorylated on tyrosine-15, indicating that the cyclin B-cdc2 complex was catalytically inactive. Maximum accumulation of these tyrosine phosphorylated forms (T_{14}-Y_{15}-T_{161} form and Y_{15}-T_{161} form) of cyclin B-cdc2 occurred in cells treated with AA 60 min prior to treatment with RGS and the levels of these forms declined thereafter.

Effects of DHA and BSO on Cell Cycle Arrest

AA can be transported into cells by two distinct mechanisms [Welch et al., 1993, 1995; Vera et al., 1993, 1995]. While AA can be transported into fibroblasts by a high affinity sodium dependent co-transporter system, the majority of AA is oxidized extracellularly to DHA and transported into the cell by glucose transporters. Once internalized, DHA is rapidly reduced to AA by a glutathione (GSH)-dependent mechanism. Our initial studies demonstrated that DHA treatment of cells also induced transient arrest at the G_2/M DNA damage checkpoint during oxidative stress (Fig. 4d), but under these conditions it was not possible to determine whether arrest was induced by AA or DHA. To determine whether AA or DHA was enhancing cell cycle arrest, GSH was depleted in cells using BSO [Griffith, 1982]. In the absence of oxidative stress, GSH depletion had no effect of the progression of AS52 cells through the cell cycle (Fig. 4e). However, during oxidative stress there was an increased accumulation of glutathione-depleted cells in S and G_2/M (Fig. 4f) when compared to untreated cells (Fig. 4a) and cells treated with RGS (Fig. 4b). Conversely, treatment of glutathione-treated cells with DHA prior to treatment with RGS resulted in enhanced accumulation of cells at G_2/M when compared to controls (Fig. 4g).

These results, summarized in Table II, suggest that the enhanced cytotoxicity observed in cells co-treated with AA and RGS was due to DHA or one of its degradative products. This was confirmed by determining the effect of GSH depletion on the protective effect of AA. BSO treatment of AS52 cells was not cytotoxic and there was only a slight increase in cytotoxicity when BSO-treated cells were treated with RGS (Table III). However, treatment of BSO-treated cells with AA or DHA prior to RGS treatment resulted in enhanced cytotoxicity of the cells during oxidative stress.

TABLE II. Effect of BSO, DHA, and RGS on Cell Cycle Progression*

Treatment	Percentage of cells		
	G_0/G_1	S	G_2/M
Untreated	35.7	61.4	3.0
RGS	42.7	45.5	11.8
DHA	36.0	56.3	7.7
DHA (2 hr) + RGS	14.9	32.7	52.4
BSO	24.6	69.5	5.9
BSO + RGS	28.9	50.6	20.5
BSO + DHA + RGS	11.9	59.0	29.1
Nocodazole	8.3	27.0	69.7

*AS52 cells were treated with BSO (0.01 M) for 24 hr prior to treatment with DHA (50 μM, 2 hr) and RGS. Cells were stained with propidium iodide and examined by flow cytometry as described in Materials and Methods. Controls cells were untreated, treated with BSO for 24 hrs, treated with RGS for 20 min, or treated with 50 μM DHA for 2 hr.

TABLE III. Modulation of the Protective Effects of Ascorbic Acid During Oxidative Stress by Buthionine Sulfoximine*

Treatment	Survival (%)
None	100.0 ± 15.2
RGS	66.7 ± 8.2
AA	97.7 ± 6.1
DHA	96.6 ± 7.8
BSO	98.3 ± 6.7
BSO and RGS	52.7 ± 7.0
BSO, AA and RGS	5.2 ± 2.7
BSO, DHA and RGS	4.4 ± 1.6

[a]AS52 cells were treated with BSO (0.01 M), AA (50 μM), DHA (50 μM), and/or RGS as described in Figures 1 and 4. Cells were treated with either AA or DHA 2 hr prior to treatment with RGS. Cytotoxicity was determined as described in Materials and Methods. The cloning efficiency of untreated cells ranges from 85–93%. Values represent the average ± the standard deviation of a minimum of three experiments.

DISCUSSION

The results of this study demonstrate that the protective effect of AA during periods of oxidative stress is due to its free-radical scavenging properties and also to its ability to enhance cell cycle arrest at the G_2/M DNA damage checkpoint. Our data also demonstrate the complexity of the checkpoint arrest mechanism — it involves the intracellular accumulation of AA, AA's stability, and at least two "signals": DHA and DNA damage. We developed two models to explain our data and these models are based on the features that in AS52 cells AA is accumulated as DHA, which is immediately reduced to AA, and that the half-life of AA is approximately 20 hr, while the half-life of DHA is approximately 0.5 hrs. In the absence of oxidative stress, AA accumulates in cells and is metabolized. While DHA is formed under these conditions, there is no cell cycle arrest,

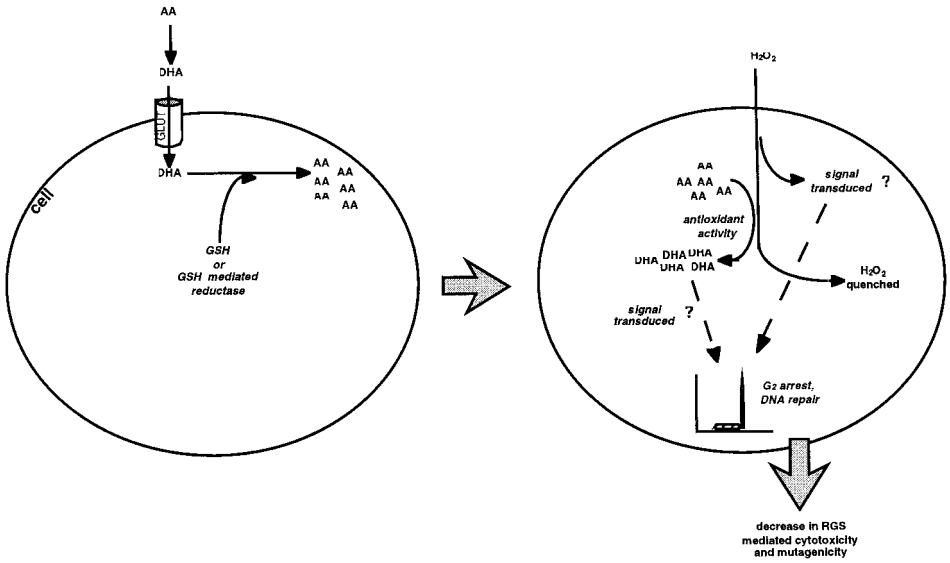

Fig. 5. Model depicting events occurring in AS52 cells treated with AA prior to treatment with RGS.

since the DNA damage signal is absent. Conversely, in AS52 cells treated with AA prior to treatment with RGS, DHA is accumulated and rapidly reduced to AA. This establishes a reduced environment in the cells. When these cells are treated with RGS, H_2O_2 damages cellular macromolecules including DNA (DNA damage signal) and AA is oxidized by H_2O_2, creating the DHA signal (Fig. 5). In this model, cellular damage is reduced by the free-radical scavenging properties of AA (decreased cytotoxicity). DNA damage is reduced by the free-radical scavenging properties of AA and by the induction of cell cycle arrest. This allows DNA repair to occur and results in decreased mutagenicity [Bijur et al., 1997].

Conversely, in cells co-treated with AA and RGS, DHA is accumulated, but because of the oxidative stress it is not reduced or reduced to a limited degree to AA (Fig. 6). Therefore, there are increased levels of H_2O_2 in these cells when compared to cells treated with AA prior to treatment with RGS. This was demonstrated by in situ histochemical-image analysis [Bijur et al., 1997]. The H_2O_2 causes the oxidation of cellular macromolecules and possibly DHA. The oxidation/metabolism of DHA results in the formation of additional metabolites that cause cellular damage. This is suggested by our studies which demonstrate that treatment of cells with BSO, AA/DHA, and RGS results in enhanced cytotoxicity when compared to controls. Cell cycle arrest also occurs in cells co-treated with AA or DHA and RGS, because the DHA and DNA damage signals are present. Then why is there enhanced mutagenesis in cells co-treated with AA and RGS? DNA damage is occurring because of the formation of H_2O_2 and while cell cycle arrest occurs, the level of cell cycle arrest in this population is not as great when compared to cell populations treated with AA prior to treatment with RGS. Therefore, in this cell population there

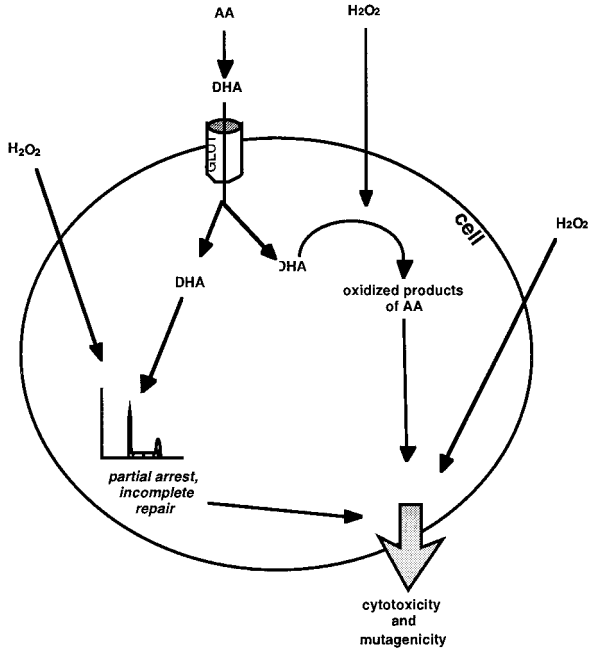

Fig. 6. Model depicting events occurring in AS52 cells co-treated with AA prior to treatment with RGS.

is a higher probability that DNA damage is fixed in the DNA. Thus, the enhanced mutation frequency observed in cells co-treated with AA and RGS [Bijur et al., 1997] reflects not only the increased DNA damage, but also the increased cytotoxicity which occurs in cells co-treated with AA and RGS.

These models are consistent with the data presented in this study and our previous study concerning AA [Bijur et al., 1997]. However, how does AA, or more precisely, DHA, effect cell cycle progression? Activation of the cyclin

B-cdc2 complex requires the sequential dephosphorylation of threonine-14 and tyrosine-15, the only tyrosine residue phosphorylated on cdc2, by the dual-specificity phosphatase cdc25C [Kumagai and Dunphy, 1991; Millar et al., 1991; Hoffmann et al., 1992; Borgne and Meijer, 1996]. Our results demonstrate that following treatment of AS52 cells with AA and RGS, both forms of cdc2 complexed to cyclin B were phosphorylated on tyrosine-15, indicating that the cyclin B-cdc2 complex is inactive. These results suggest that DHA is modulating, either directly or indirectly, the activity of cdc25C.

There is increasing evidence suggesting that the redox (oxidation/reduction) status regulates cellular functions. Reactive oxygen species (ROS) such as superoxide anion and the hydroxyl radical and reactive oxygen intermediates (ROI), such as H_20_2, are formed during normal cellular metabolism and following the exposure of cells to certain environmental agents. Overproduction of ROI/ROS can be detrimental to a cell by causing membrane peroxidation, protein oxidation, and DNA damage. Conversely, ROS/ROI also act as mediators in certain signal transduction pathways [Baas and Berk, 1995; Sandaresan et al., 1995; Guyton et al., 1996; Irani et al., 1997] and they can regulate gene transcription by activating various transcription factors [Baeuerle and Henkel, 1994; Schenk et al., 1994; Anderson et al., 1994; Galter et al., 1994; Stauble et al., 1994; Sen and Packer, 1996; Flohe et al., 1997]. Our results provide evidence that the redox status may be important in regulating cell cycle progression during oxidative stress. While additional studies are necessary to define the mechanism by which AA/DHA regulates cdc25C, our results demonstrate a novel mechanism by which AA protects cells from damage due to oxidative stress and a unique redox mechanism involved in cell cycle progression.

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