ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 1 & 2, 2005

Original Research Communication

Vitamin C-Induced Loss of Redox-Dependent Viability in Lung Microvascular Endothelial Cells

SARADHADEVI VARADHARAJ,[1] TONYA WATKINS,[2] ARTURO J. CARDOUNEL,[1] JOE G.N. GARCIA,[2] JAY L. ZWEIER,[1] PERIANNAN KUPPUSAMY,[1] VISWANATHAN NATARAJAN,[2] and NARASIMHAM L. PARINANDI[1]

ABSTRACT

Recent clinical trials have shown that vitamin C, at pharmacological concentrations (milligram to approximately gram), upon infusion into circulation, modulates vasodilation and vascular tone in humans. This also results in the elevated concentrations of vitamin C in circulation in the millimolar range. Here, it was hypothesized that vitamin C at pharmacological concentrations (millimolar) would induce oxidative stress and cause loss of redox-dependent cell viability in vascular endothelial cells (ECs). To test the hypothesis, bovine lung microvascular ECs (BLMVECs) in monolayer cultures were exposed to vitamin C (0–10 m*M*) for different time periods (0–2 h). Electron paramagnetic resonance spectroscopy revealed the intracellular formation of ascorbate free radical in a dose- and time-dependent fashion. Vitamin C also induced formation of intracellular reactive oxygen species in a dose-dependent fashion. It was observed that vitamin C induced morphological alterations and loss of cell viability in a dose- and time-dependent fashion, as measured by light microscopy and Alamar Blue redox cell viability assay, respectively. Vitamin C analogues failed to induce such changes. Vitamin C depleted cellular GSH levels in a dose-dependent fashion, suggesting that vitamin C altered thiol-redox status in BLMVECs. Antioxidants, intracellular iron chelator, and catalase protected cells against vitamin C-induced loss of redox-dependent cell viability, confirming the role of hydrogen peroxide and iron during redox cycling of vitamin C. These results, for the first time in detail, established that vitamin C at pharmacological doses induced oxidative stress and loss of redox-dependent cell viability in microvascular ECs. *Antioxid. Redox Signal.* 7, 287–300.

INTRODUCTION

VITAMIN C (ASCORBIC ACID), an essential water-soluble vitamin, is well known for its antiscorbutic and antioxidant functions in humans (16, 41, 42, 47). Apart from its essential role in human nutrition, vitamin C has occupied an important place in preventive medicine to safeguard health from several pathological conditions, such as cancer, infections, viral respiratory illnesses, common cold, and above all, cardiovascular diseases. Vitamin C, as an electron donor and a redox-active compound, participates as a cofactor in many enzymatic reactions, including the hydroxylation of collagen, synthesis of carnitine, synthesis of noradrenaline, metabolism of tyrosine, and nitric oxide synthesis (17, 41). Also, vitamin C is a powerful antioxidant that participates in many nonenzymatic reactions, thus preventing oxidation of low-density lipoproteins, alleviates oxidative stress in the stomach, and increases iron absorption (47). Oxidative stress has been im-

[1]Lipid Signaling and Lipomics Laboratory, Division of Pulmonary, Critical Care and Sleep Medicine, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH.
[2]Division of Pulmonary and Critical Care Medicine, Johns Hopkins University, Baltimore, MD.

plicated in several diseases, including cardiovascular diseases, and antioxidant intervention (nutritional supplementation) has been shown to alleviate those pathological conditions (12). A correlation has been established between low vitamin C concentrations in the serum and peripheral arterial disease that is associated with inflammation and severity of atherosclerosis (39). Vitamin C has been called "A Cure for Miracles," and subclinical deficiency of the vitamin may be of common occurrence in humans (35). Thus, a deficiency of vitamin C in humans with cardiovascular disorders necessitates a proper vitamin C supplemental intervention in those patients.

Recent clinical trials have provided compelling evidence that vitamin C at pharmacological concentrations, upon infusion into circulation, modulates vasodilation and vascular tone in humans. Alternate therapy practitioners and some clinicians are in favor of vitamin C infusion at megadoses (~grams) into circulation to treat cancer in humans (46). Infusion of pharmacological concentrations (milligrams to gram) of vitamin C raises the circulating levels of the vitamin to millimolar levels in humans, and the use of supraphysiological doses of vitamin C in clinical trials as a therapeutic agent to treat cardiovascular diseases is gaining attention (6, 26, 29, 32, 34, 35, 70). Vitamin C, in addition to functioning as an antioxidant, acts as a prooxidant and generates ascorbate free radical (AFR) and reactive oxygen species (ROS) in biological systems in the presence of transition metal ions (Fe^{2+}) (1, 10, 18, 21, 27, 28). The endothelium, a semipermeable barrier that plays a pivotal role in the maintenance of vascular integrity and function, is a prime target for the high levels of circulating prooxidant vitamin C, presumably leading to altered integrity of the endothelium and ultimately of the vessel. It has been reported that vitamin C, upon infusion into blood at a dose of 10 mg/min for 120 min, improved the impairment of endothelial function in smokers (32). Cigarette smoke products have been implicated in the alterations of pulmonary vascular endothelium, leading to pulmonary hypertension in chronic obstructive pulmonary disease (5). Hence, in the current study, we have chosen to use the bovine lung microvascular endothelial cells (BLMVECs) as the most appropriate model endothelial cell (EC) system. Although the vasomodulatory therapeutic effects of vitamin C have been advocated, the adverse effects of this molecule on vascular endothelium, at pharmacological doses, have not been well studied. Therefore, in the current study, we have investigated vitamin C-induced oxidative stress and the associated alterations in redox-dependent cell viability in the BLMVECs.

The results of the current study, for the first time, revealed (a) formation of intracellular AFR and ROS in a dose- and time-dependent fashion, (b) morphological alterations and loss of redox-dependent cell viability, (c) depletion of cellular thiols [glutathione(GSH)], and (d) protection against loss of redox-dependent cell viability by chelation of intracellular iron, antioxidants, and catalase in BLMVECs exposed to pharmacological doses of vitamin C. These results further established that vitamin C at pharmacological doses induced oxidative stress and loss of redox-dependent cell viability in the lung microvascular ECs in culture.

MATERIALS AND METHODS

Reagents

Minimum essential medium (MEM), L-ascorbic acid, L-ascorbyl-2-phosphate, L-ascorbyl-2-sulfate, nonessential amino acids, trypsin-EDTA, Na_2-EDTA, diethylenetriaminepentaacetic acid (DTPA), *N*-acetylcysteine (NAC), δ-gluconolactone, penicillin–streptomycin, fetal bovine serum (FBS), bovine liver catalase, bovine erythrocyte superoxide dismutase (SOD), and propyl gallate were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dehydroascorbic acid, SOD mimetics (MnTBAP and MnTMPyP), and desferal were obtained from Calbiochem (San Diego, CA, U.S.A.). BLMVECs (passage 4) were purchased from Cell Systems (Kirkland, WA, U.S.A.). Cell growth supplement was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Alamar Blue™ reagent was obtained from Biosource International (Camarillo, CA, U.S.A.). 6-Carboxy-2′,7′-dichlorodihydroxyfluorescein diacetate dicarboxy methyl ester (DCFDA) and Amplex Red kit were purchased from Molecular Probes (Eugene, OR, U.S.A.). Hanks' buffer containing potassium phosphate (0.44 m*M*), potassium chloride (5.37 m*M*), dibasic sodium phosphate (0.34 m*M*), sodium chloride (136.89 m*M*), and D-glucose (5.55 m*M*) was purchased from GIBCO (Carlsbad, CA, U.S.A.). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) spin trap was obtained from Dojindo Laboratories (Gaithersburg, MD, U.S.A.).

EC culture

BLMVECs were cultured in MEM containing FBS (10%), antibiotics (1%), nonessential amino acids (1%), and endothelial growth supplement, maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2, and grown to contact-inhibited monolayers with typical cobblestone morphology (45). Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh medium, and subcultured in 35-mm and 60-mm sterile dishes in complete medium to ~95% confluence under 95% air/5% CO_2 for exposure to vitamin C (L-ascorbic acid) and desired pharmacological agents. MEM containing vitamin C and other pharmacological agents was carefully adjusted to pH 7.4 for cellular treatments. The cells from passages 6–13 were used in all the experiments.

Detection of intracellular ROS generation

Intracellular generation of ROS was detected by fluorescence microscopy based on the oxidation of DCFDA (52). The assay is based on the principle of passive diffusion of the nonpolar and nonfluorescent dye into the cells followed by hydrolysis by intracellular esterases to a nonfluorescent derivative, dichlorofluorescein, and subsequent oxidation into a fluorescent dye by the intracellular ROS. BLMVECs (~95% confluence) were grown on cover slips and loaded with DCFDA (10 μ*M*) for 30 min, following which the medium containing DCFDA was removed and the cells were treated with vitamin C (0–10 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end

of the incubation period, the cells were washed with phosphate-buffered saline (PBS; 37°C) and examined under a Nikon Eclipse TE 300 microscope with excitation and emission set at 490 and 530 nm, respectively. Fluorescence of oxidized DCFDA in cells was captured with a Sony digital DKC 5000 camera.

Assay of endothelial injury and cytotoxicity

The morphology of BLMVECs following the exposure to vitamin C was assessed by light microscopy (52). Vitamin C-induced loss of cell viability was assayed by the Alamar Blue reduction method utilizing the Alamar Blue cell viability assay reagent (Biosource) as per the manufacturer's recommendations. In principle, this method is based on the spectrophotometric assay of bioreduction of the active ingredient, resazurin, a redox-sensitive dye present in Alamar Blue reagent. BLMVECs, cultured in 35-mm dishes (~95% confluence), were exposed to vitamin C (0–10 m*M*) for different time periods in the absence and presence of antioxidants and pharmacological inhibitors wherever desired, following which the medium was removed; the cells were washed once with 1.0 ml of PBS (37°C) and incubated with 1 ml of fresh MEM containing 100 μl of Alamar Blue reagent for 3 h under humidified 95% air/5% CO_2 atmosphere at 37°C. The metabolic reduction of redox probe (resazurin) of Alamar Blue by cells was measured spectrophotometrically at 570 and 600 nm on a Molecular Devices Spectra Max 190 UV-visible plate reader according to the manufacturer's instructions. Vitamin C alone, at any tested concentration, did not show any effect on the extent of cellular metabolic reduction of Alamar Blue reagent. The extent of Alamar Blue reduction was normalized to the corresponding untreated control cells and expressed as percent cell viability.

Determination of hydrogen peroxide (H_2O_2) by Amplex Red assay

The formation of H_2O_2 in the medium and cells was determined by fluorescence method (52) using the Amplex Red H_2O_2 assay kit (Molecular Probes). BLMVECs grown in 35-mm dishes (~95% confluence) were exposed to vitamin C (0–10 m*M*) under a humidified atmosphere of 95% air/5% CO_2 for 120 min in 1.0 ml of Hanks' buffer without phenol red. Phenol red-containing medium (MEM) was not chosen for this assay to avoid the interference caused by phenol red in the fluorimetric assay of H_2O_2. At the end of incubation, the medium was removed from the cells and centrifuged at 4,000 *g* for 5 min to remove any floating cells. The cells attached to the substratum were detached with a cell scraper in 500 μl of Hanks' buffer and sonicated three times with a probe sonicator at a setting of 5 on ice. Fluorescence of the medium and cell lysates was measured with appropriate blanks on a fluorescence plate reader (Molecular Devices, Spectra Max Gemini XS) with excitation and emission set at 560 and 590 nm, respectively. The concentrations of H_2O_2 generated in the medium and cells were calculated from the standard curve prepared with known concentrations of H_2O_2 and were expressed as micromolar per milligram of protein.

Electron paramagnetic resonance (EPR) spectroscopy

BLMVECs grown in 35-mm dishes (~95% confluence) were exposed to different concentrations of vitamin C (0–10 m*M*) in the absence and presence of 50 m*M* DMPO in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C for the required lengths of time (0–120 min). At the end of incubation, the medium containing vitamin C was removed, cells were washed twice with PBS, scraped in 1 ml of PBS, and transferred into a flat cell. Removal of incubation medium and washing cells with PBS will ensure only the detection of intracellular radicals. EPR spectra were recorded in flat cells at room temperature with a Bruker ER 300 spectrometer with a TM-110 cavity operating at X-band (9.78 GHz) using the following parameters: microwave power, 20 mW; modulation amplitude, 0.5 G; modulation frequency, 100 kHz; receiver gain, 5×10^5; after loading the samples into the TM-110 cavity (78).

GSH determination

Intracellular concentrations of GSH in BLMVECs grown in 60-mm dishes (~95% confluence), following exposure to vitamin C (0–10 m*M*) in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C for 120 min, were determined by the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)-coupled spectrophotometric assay of the cell lysates (51). The levels of GSH were calculated from a standard curve prepared with GSH and expressed as micrograms per milligram of cellular protein.

Statistical analysis

All experiments were done in triplicate. Data are expressed as means ± SD. Statistical analysis was carried out by ANOVA using SigmaStat (Jandel). The level of statistical significance was taken as $p < 0.05$.

RESULTS

AFR formation in vitamin C-treated BLMVECs

Vitamin C undergoes oxidation and generates AFR in physiological milieu (55), which has been used as an index of oxidative stress in biological systems (10, 13, 61). As shown in Fig. 1A, exposure of BLMVECs to 3–7 m*M* vitamin C for 120 min, compared with cells exposed to 1 m*M* vitamin C or vehicle alone, generated AFR as detected by EPR. Although BLMVECs exposed to 5 and 7 m*M* vitamin C for 120 min showed a striking generation of the AFR, the amplitude of the radical was not greater in those cells as compared with cells exposed to 3 m*M* vitamin C (Fig. 1A). The formation of AFR in BLMVECs treated with vitamin C was also time-dependent. BLMVECs exposed to 5 m*M* vitamin C clearly revealed the formation of AFR as early as 30 min of incubation, and further exposure of the cells to the same concentration of vitamin C at 60 and 120 min resulted in an increase in the amplitude of AFR (Fig. 1B). Attempts to detect the ROS [super-

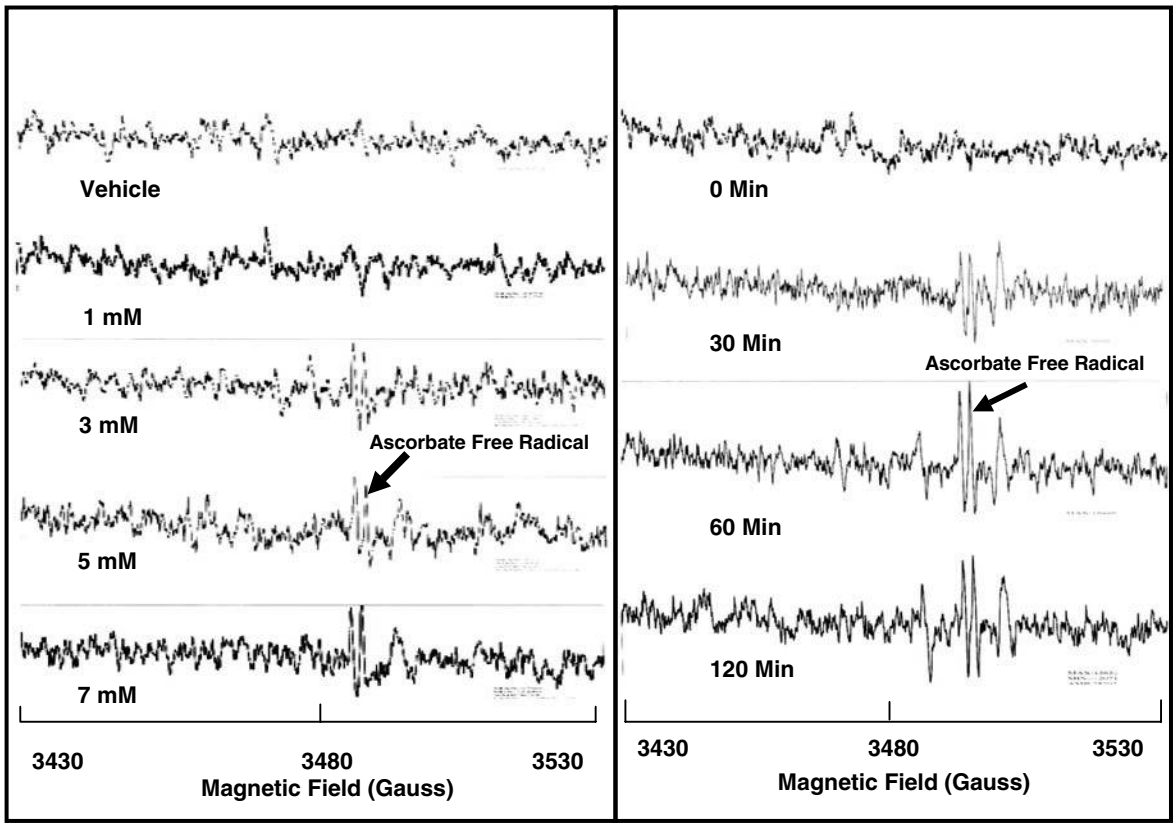

FIG. 1. AFR formation in vitamin C-treated BLMVECs. BLMVECs (~95% confluence) grown in 35-mm dishes were treated with different concentrations of vitamin C (0–7 m*M*) for 120 min for the dose-response study **(A)** and with 5 m*M* vitamin C for different periods of time (0–120 min) for the time-course study **(B)** in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. EPR spectra were recorded as described in Materials and Methods. Each spectrum presented is a representative of three individual spectra obtained under similar conditions.

oxide anion radical ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$)] generated by vitamin C in BLMVECs by EPR spectroscopy using the DMPO spin trap were unsuccessful. This may indicate that the level of $O_2^{-\bullet}$ generated in the system was below the detection sensitivity of the spin trap. These data showed that exposure of BLMVECs to vitamin C led to the formation of AFR in a dose- and time-dependent fashion.

Vitamin C induces generation of intra- and extracellular ROS

Earlier studies have shown that vitamin C, a redox-active prooxidant, induces oxidative stress in several systems (71); however, no data are available on vitamin C-mediated production of ROS in vascular ECs. Fluorescence microscopic examination of BLMVECs loaded with the ROS fluorophore, DCFDA, following exposure to vitamin C (5 m*M*) for 120 min showed a marked generation of the intracellular ROS at 15 min, which increased with time up to 120 min (Fig. 2A–F). Cells preloaded with DCFDA and incubated for 120 min in the absence of vitamin C did not exhibit noticeable fluorescence (Fig. 2G). Next, we quantified levels of H_2O_2 formed upon exposure of BLMVECs to vitamin C using Amplex Red assay. As shown in Fig. 3, exposure of BLMVECs to vitamin C increased the formation of extracellular and intracellular H_2O_2, in a dose-dependent manner, as compared with cells treated with vehicle alone. When BLMVECs were exposed to 1 m*M* vitamin C for 120 min, the levels of H_2O_2 formed in the medium and cells were induced six-fold (0.565 μ*M*/mg of protein) and 10-fold (11.31 μ*M*/mg of protein), respectively, as compared with their corresponding controls, which yielded 0.05 μ*M*/mg of protein and 1.86 μ*M*/mg of protein H_2O_2 in the medium and cells, respectively (Fig. 3). BLMVECs exposed to 3 m*M* vitamin C showed an eight-fold (1.03 μ*M*/mg of protein) and 10-fold (18.12 μ*M*/mg of protein) formation of H_2O_2 in the medium and cells, respectively as compared with their corresponding controls (Fig. 3). The data revealed both the extracellular and intracellular formation of H_2O_2 following exposure of BLMVECs to vitamin C, wherein the formation of H_2O_2 in the cells was higher than that observed in the medium.

Effect of vitamin C on redox-dependent loss of cell viability and morphology

As oxidative stress causes alterations in the cellular redox status and loss of cell viability (23), the redox-dependent cell viability in BLMVECs upon exposure to vitamin C was in-

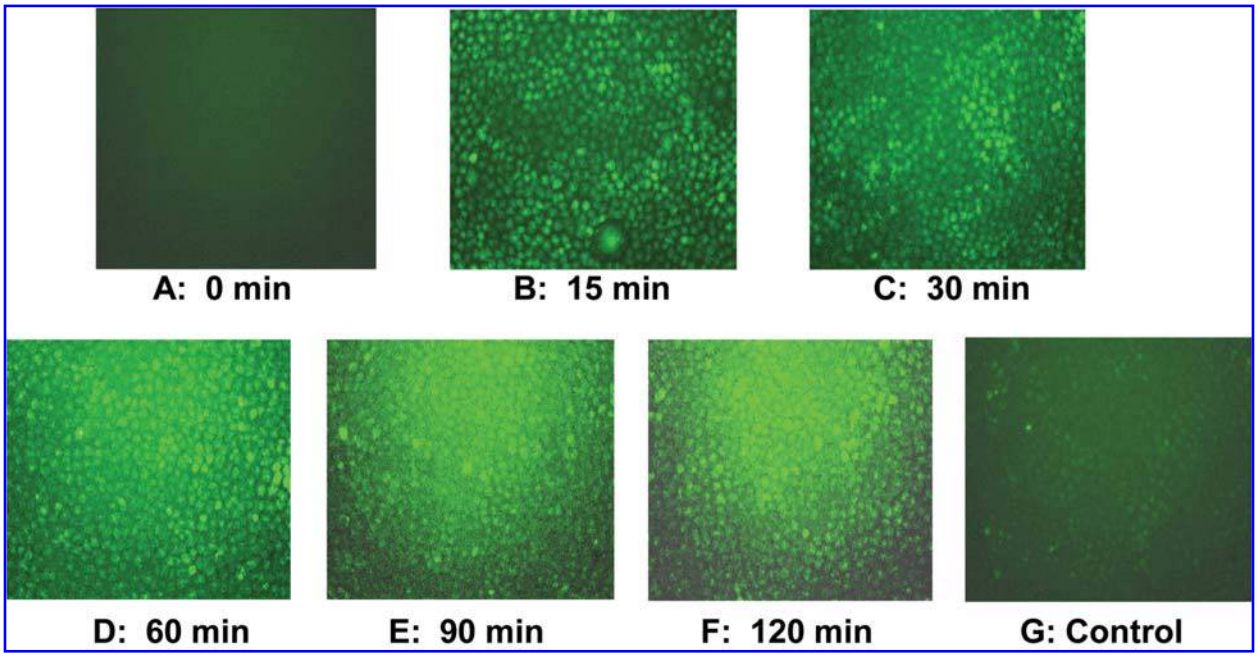

FIG. 2. Vitamin C induces generation of intracellular ROS. BLMVECs (~95% confluence) grown on cover slips were loaded with 10 μ*M* DCFDA for 30 min in complete medium and treated with vitamin C (5 m*M*) for 120 min for different lengths of time (0–120 min) in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C **(A–F)**. Cells preloaded with DCFDA as described before were incubated for 120 min in the absence of vitamin C, which served as control **(G)**. At the end of incubation, the formation of ROS in cells was examined under a fluorescence microscope as described in Materials and Methods. Each panel is a representative of three individual experiments conducted under similar conditions.

vestigated using the cell viability indicator (Alamar Blue reagent) that contains a cellular redox indicator, resazurin, which is metabolized solely by the redox-dependent enzymes (64, 72). As shown in Fig 4, vitamin C in a dose-dependent manner induced a significant loss of cell viability in BLMVECs. At 3, 5, 7, and 10 m*M* concentrations of vitamin C, the index of redox-dependent cell viability (% Alamar Blue reduction) was 80%, 55%, 44%, and 22%, respectively (Fig. 4). Furthermore, vitamin C at 3, 5, and 10 m*M* concentrations caused a significant loss in the redox-dependent cell viability in a time-dependent fashion. At 120 min of incubation of BLMVECs with 3, 5, and 10 m*M* vitamin C, the redox-dependent cell viability was 50%, 41%, and 45%, respectively. Vitamin C at 1 m*M* concentration did not appear to

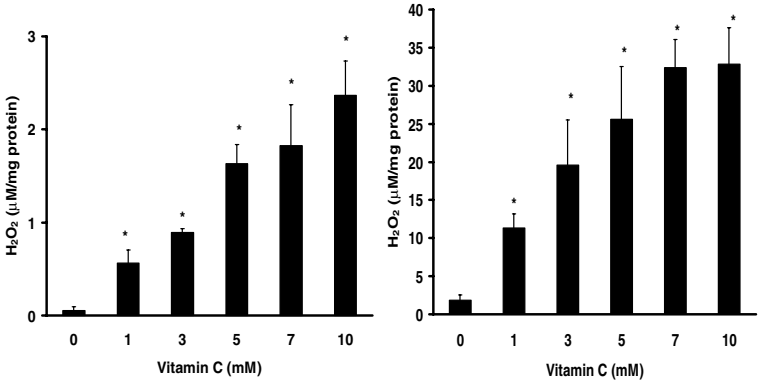

FIG. 3. Vitamin C induces extracellular and intracellular formation of H_2O_2. BLMVECs (~95% confluence) grown in 35-mm dishes were treated with different concentrations of vitamin C (0–10 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the formation of H_2O_2 in the medium **(A)** and cells **(B)** was determined fluorimetrically using the Amplex Red H_2O_2 assay kit (Molecular Probes) as described in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with vehicle-treated controls.

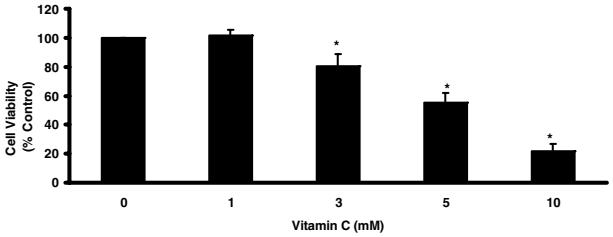

FIG. 4. Vitamin C induces loss of redox-dependent cell viability in a dose-dependent fashion. BLMVECs (~95% confluence) grown in 35-mm dishes were treated with different concentrations of vitamin C (0–10 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with vehicle-treated controls.

cause a significant and marked loss of redox-dependent cell viability from 0–120 min of exposure (Fig. 5). These results confirmed that vitamin C caused a marked and significant (a) decrease in the cellular redox activity and (b) loss of redox-dependent cell viability in a dose- and time-dependent fashion in BLMVECs. The data further suggested the involvement of cellular redox status and oxidative stress in the vitamin C-induced loss of cell viability in BLMVECs.

Vitamin C alters cell morphology

Earlier studies have reported that oxidative stress causes alterations in the cell morphology (7). Therefore, the vitamin C-induced alterations of cellular morphology were studied by light microscopy. BLMVECs treated with vitamin C at 5, 7, and 10 mM concentrations for 120 min exhibited marked alterations in their cell morphology with considerable gaps between cells and formation of filopodia and lamellipodia (Fig. 6). At concentrations of 3 m*M* or less, vitamin C did not alter the cell morphology or shape in BLMVECs. These results demonstrated that vitamin C at pharmacological doses caused alterations in the cell morphology in BLMVECs.

Analogues of vitamin C fail to cause loss of redox-dependent cell viability

Next we investigated the potencies of vitamin C and its analogues (Fig. 7) in causing the loss of redox-dependent cell viability in BLMVECs. As shown in Fig. 8, BLMVECs exposed to vitamin C (5 m*M*) for 120 min showed a significant loss in redox-dependent cell viability (decrease in cellular redox), whereas all the other chosen analogues of vitamin C failed to cause such a loss of cellular viability. The results showed that (a) none of the tested analogues of vitamin C caused the loss of redox-dependent cell viability, (b) δ-gluconolactone (also known as D-gluconic acid lactone, the precursor of vitamin C with similar molecular weight) did not cause any loss of redox-dependent cell viability, (c) L-ascorbyl-2-sulfate and L-ascorbyl-2-phosphate did not induce any loss of redox-dependent cell viability, and (d) the ene-diol nature of vitamin C was necessary to induce the loss of redox-dependent cell viability in BLMVECs (Fig. 8). Both vitamin C-2-sulfate and vitamin C-2-phosphate derivatives have been shown to be nontoxic to the P388D1 murine leukemia cells (57). Our results are in agreement with these findings and further suggested that the lack of cytotoxicity-inducing effect of those two derivatives in BLMVECs may be attributed to the low activity or absence of the exofacial phosphatases and sulfatases in the cell (43), which hydrolyze the phosphate and sulfate moieties in those derivatives, rendering ascorbic acid physiologically active. This study also established that vitamin C-induced loss of redox-dependent cell viability in BLMVECs was not due to the osmotic shock exerted by vitamin C at pharmacological doses tested as no such toxic response was observed when the cells were treated with the iso-structural molecules such as δ-gluconolactone, dehydroascorbic acid, and other analogues of vitamin C at the same concentrations as vitamin C.

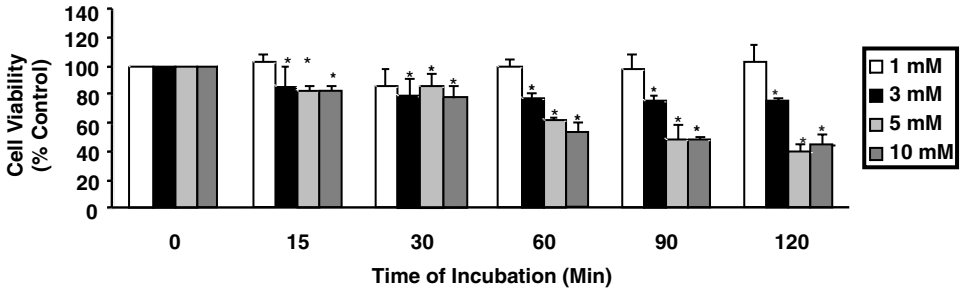

FIG. 5. Vitamin C induces loss of redox-dependent cell viability in a time-dependent manner. BLMVECs (~95% confluence) grown in 35-mm dishes were treated with different concentrations of vitamin C (1–10 m*M*) for different periods of time (0–120 min) in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the cells incubated for 0 min.

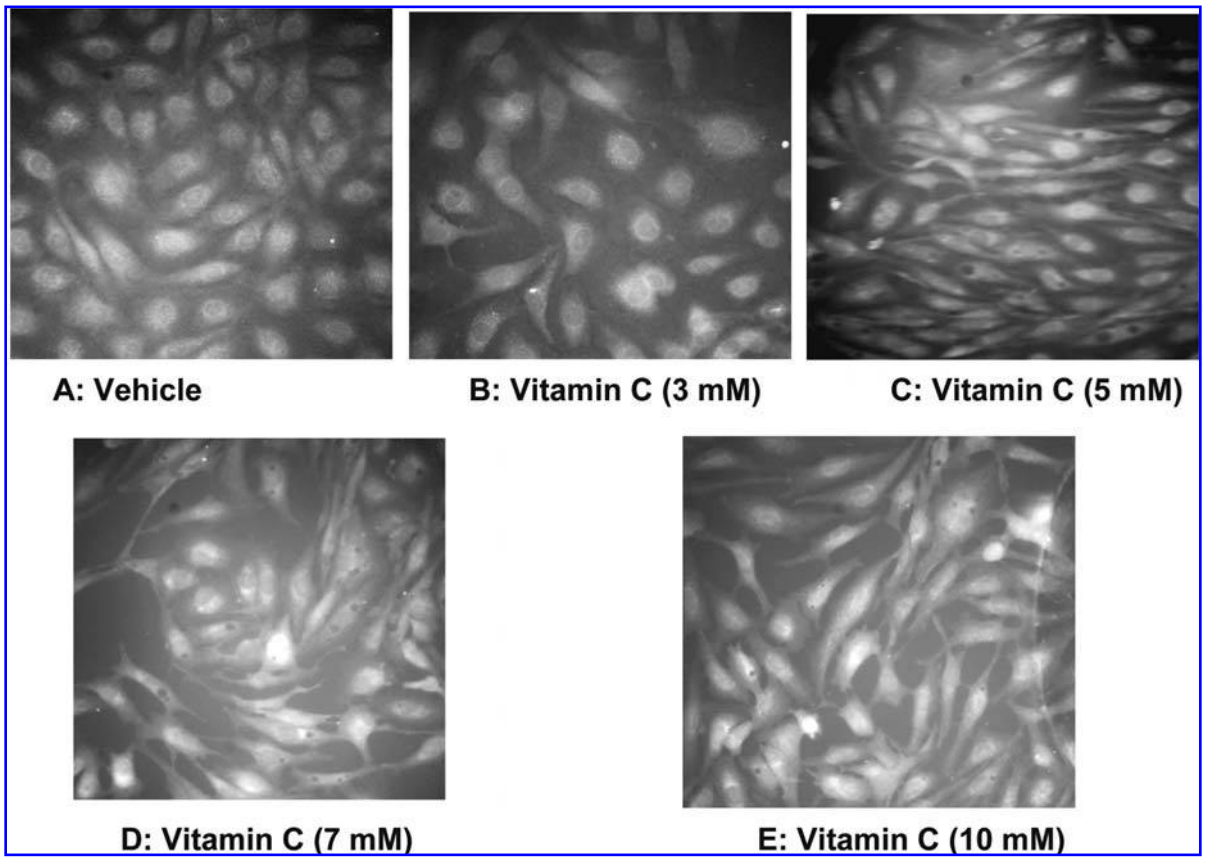

FIG. 6. Vitamin C alters cell morphology. BLMVECs (~80% confluence) grown on cover slips were exposed to vitamin C (0–10 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the cell morphology was examined under a light microscope.

Vitamin C depletes cellular GSH

Oxidants and oxidative stress are known to deplete the cellular thiol-redox pools, including GSH (25), another useful and accepted index of oxidative stress. Hence, the levels of GSH in BLMVECs following exposure to vitamin C (0–10 m*M*) at 120 min were determined. Vitamin C caused a significant depletion of GSH (50% depletion) even at 1 m*M* con-

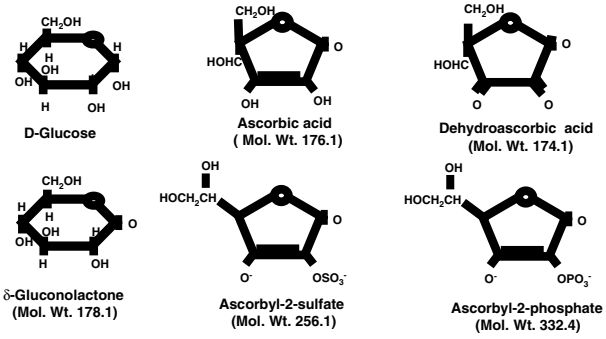

FIG. 7. Molecular structures of vitamin C and its analogues.

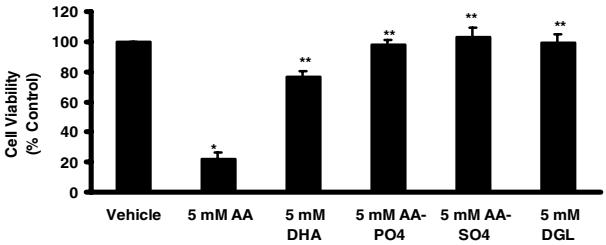

FIG. 8. Analogues of vitamin C fail to cause loss of cell viability. BLMVECs (~95% confluence) grown in 35-mm dishes were treated with 5 m*M* concentrations of vitamin C (AA) and its analogues (dehydroascorbic acid, DHA; ascorbyl-2-phosphate, AA-PO4; ascorbyl-2-sulfate, AA-SO4; δ-gluconolactone, DGL) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells; **significantly different at $p < 0.05$ as compared with the vitamin C-treated cells.

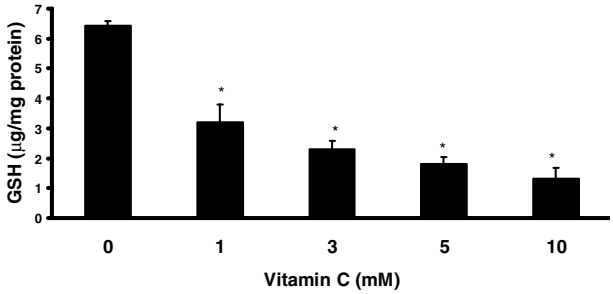

FIG. 9. Vitamin C depletes cellular GSH. BLMVECs (~95% confluence) grown in 60-mm dishes were treated with different concentrations of vitamin C (0–10 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the cellular GSH levels were determined spectrophotometrically as described in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells.

centration, and at 3, 5, and 10 m*M* concentrations of vitamin C, further depletion of intracellular GSH to 64%, 72%, and 80% in BLMVECs at 120 min of exposure was observed (Fig. 9). These results demonstrated that vitamin C at pharmacological doses caused a significant depletion of the cellular GSH in BLMVECs.

Antioxidants protect against vitamin C-induced loss of redox-dependent cell viability

Antioxidants are known to protect against the cytotoxicity induced by oxidants and oxidative stress (44). As the preceding results of the current study established that vitamin C at pharmacological doses induced the generation of ROS and AFR, caused the loss of redox-dependent cell viability, and depleted the cellular GSH, the two well established water-soluble antioxidants, propyl gallate and NAC, were tested for their protective effects against loss of cell viability in BLMVECs. As shown in Figs. 10 and 11, propyl gallate (1 m*M*) and NAC (10 m*M*) offered a significant protection against the loss of redox-dependent cell viability in BLMVECs exposed to vitamin C (5 m*M*) for 120 min. Even 100 μ*M* propyl gallate was effective in offering almost a complete protection against the vitamin C-induced loss of redox-dependent cell viability (data not shown). These results suggest that antioxidants and thiol-replenishing agents significantly protected against the vitamin C-induced loss of redox-dependent cell viability in BLMVECs.

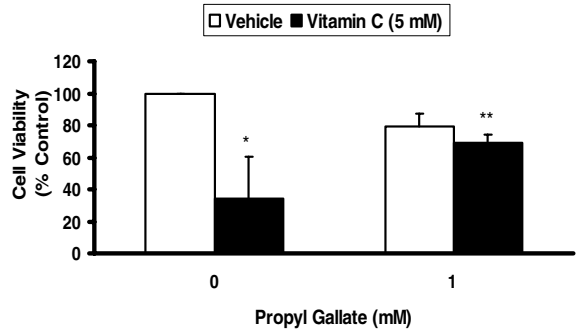

FIG. 10. Propyl gallate protects against vitamin C-induced loss of cell viability. BLMVECs (~95% confluence) grown in 35-mm dishes were pretreated with propyl gallate (1 m*M*) for 2 h to assure uptake of the antioxidant by the cells and then exposed to vitamin C (5 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells; **significantly different at $p < 0.05$ as compared with the vitamin C-treated cells.

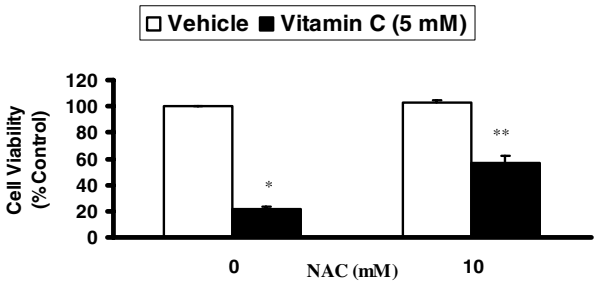

FIG. 11. NAC protects against vitamin C-induced loss of cell viability. BLMVECs (~95% confluence) grown in 35-mm dishes were pretreated with NAC (10 m*M*) for 2 h to assure uptake of the antioxidant by the cells and then exposed to vitamin C (5 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells; **significantly different at $p < 0.05$ as compared with the vitamin C-treated cells.

Iron chelator protects BLMVECs against vitamin C-induced loss of redox-dependent cell viability

The role of iron in oxidant-mediated and especially in vitamin C-induced oxidative stress has been well established (4, 38). Hence, the role of iron in vitamin C-induced loss of redox-dependent cell viability in BLMVECs was investigated in the present study. As shown in Fig. 12, the common extracellular iron chelators, EDTA (1.0 m*M*) and DTPA (1.0 m*M*), failed to offer a significant protection against the loss of redox-dependent cell viability in BLMVECs exposed to vitamin C (5 m*M*) for 120 min. On the other hand, desferal, an intracellular iron (Fe^{3+}) chelator (33), offered a marked and significant protection (2.4-fold at 1.0 m*M*) against the vitamin C-induced loss of redox-dependent cell viability in BLMVECs (Fig. 12). Desferal, even at a concentration of 0.5 m*M*, significantly offered a two-fold protection against the vitamin C-induced loss of redox-dependent cell viability in

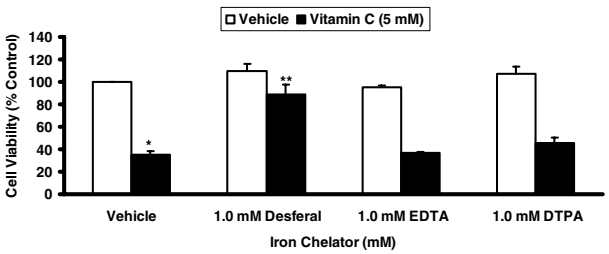

FIG. 12. Intracellular iron chelator protects against vitamin C-induced loss of cell viability. To study the role of intracellular iron in vitamin C-induced loss of cell viability, BLMVECs (~95% confluence) grown in 35-mm dishes were pretreated with desferal (1.0 m*M*) for 2 h to assure sequestration of intracellular iron by the chelator and then exposed to vitamin C (5 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. To investigate the role of extracellular iron in vitamin C-induced loss of cell viability, cells were treated with the extracellular iron chelators EDTA (1.0 m*M*) and DTPA (1.0 m*M*) in the presence of vitamin C (5 m*M*) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells; **significantly different at $p < 0.05$ as compared with the vitamin C-treated cells.

BLMVECs (data not shown). These results suggested the involvement of intracellular iron (Fe^{3+}) in the vitamin C-induced loss of redox-dependent cell viability in BLMVECs.

Catalase protects against vitamin C-induced loss of cell viability

Our results suggest that vitamin C induced generation of extra- and intracellular H_2O_2 in BLMVECs, which may be involved in loss of cell viability. Therefore, the effect of SOD, SOD mimetics, and catalase on the vitamin C-induced loss of redox-dependent cell viability in BLMVECs was studied. Addition of catalase (300 μg/ml) to the medium offered a significant protection (85%) and almost a complete protection at 500 μg/ml against the loss of redox-dependent cell viability in BLMVECs exposed to vitamin C (5 m*M*) for 120 min (Fig. 13). However, SOD and SOD mimetics, when present in the medium, failed to offer similar protection (data not shown). These results confirmed the involvement of H_2O_2 in the vitamin C-induced loss of redox-dependent cell viability in BLMVECs.

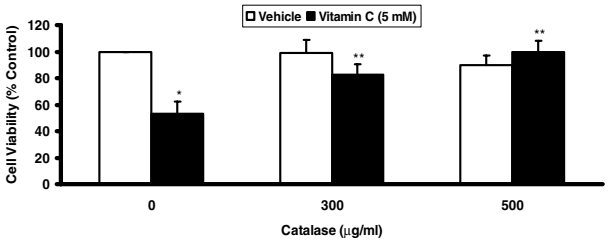

FIG. 13. Catalase protects against vitamin C-induced loss of cell viability. BLMVECs (~95% confluence) grown in 35-mm dishes were exposed to vitamin C (5 m*M*) in the presence of catalase (0, 300, and 500 μg) for 120 min in MEM under a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of exposure, the redox-dependent cell viability was determined spectrophotometrically using the Alamar Blue reagent as outlined in Materials and Methods. Results are expressed as means ± SD from three individual experiments in triplicate. *Significantly different at $p < 0.05$ as compared with the vehicle-treated cells; **significantly different at $p < 0.05$ as compared with the vitamin C-treated cells.

DISCUSSION

Infusion of pharmacological doses of vitamin C (milligrams to grams) is emerging as a therapeutic intervention against certain types of cancer and cardiovascular diseases (6, 26, 29, 32, 34, 35, 70). As the prooxidant nature of vitamin C has been well established (1, 10, 18, 21, 27, 28) and the adverse effects of the vitamin at pharmacological doses on the vascular endothelium have not been thoroughly understood, here we investigated the oxidative stress and associated loss of redox-dependent cell viability induced by vitamin C at millimolar concentrations in cultured BLMVECs. The current study, for the first time, comprehensively demonstrated the (a) formation of intracellular AFR in a dose- and time-dependent fashion, (b) formation of intracellular ROS in a dose-dependent fashion as detected by the increase in DCFDA fluorescence, (c) formation of elevated levels of H_2O_2 in the medium and cells, (d) morphological alterations and loss of redox-dependent cell viability in a dose- and time-dependent fashion, (e) depletion of cellular thiols (GSH) in a dose-dependent fashion, and (f) protection against loss of redox-dependent cell viability by intracellular iron chelator, antioxidants, and catalase in BLMVECs exposed to pharmacological concentrations (1–10 m*M*) of vitamin C.

In the extracellular environment such as plasma, vitamin C undergoes one-electron oxidation to AFR followed by spontaneous disproportionation to dehydroascorbic acid and ascorbic acid (Fig. 14) (2, 10, 42, 47). Alternatively, the AFR may recycle to ascorbic acid by the action of membrane-localized NADH-semidehydroascorbate reductase (AFR reductase) (2). Dehydroascrobic acid is transported into the cell by glucose transporters (GLUT1, GLUT3, and GLUT4) and is converted back to ascorbic acid by thioredoxin reductase or glutaredoxin, involving NADPH and GSH, whereas ascorbic acid is taken up into the cell by sodium-dependent vitamin C transporters (SVCT1 and SVCT2) (Fig. 15) (42, 47). Vitamin C has been shown to undergo autoxidation in the presence of transition metal ions (copper and iron) forming AFR and dehydroascorbic acid via two successive one-electron oxidations along with the generation of ROS ($O_2^{-\bullet}$, H_2O_2, and $^{\bullet}OH$) (Fig. 14) (68). The formation of AFR, as detected by EPR spectroscopy, has been used as a reliable indicator of the free radical-mediated myocardial ischemic and postischemic injury and a marker for the oxidative stress in cells (13, 14, 54, 59–63). Vitamin C has also been shown to generate radicals and cause oxidative stress (50, 55, 71). It has been reported

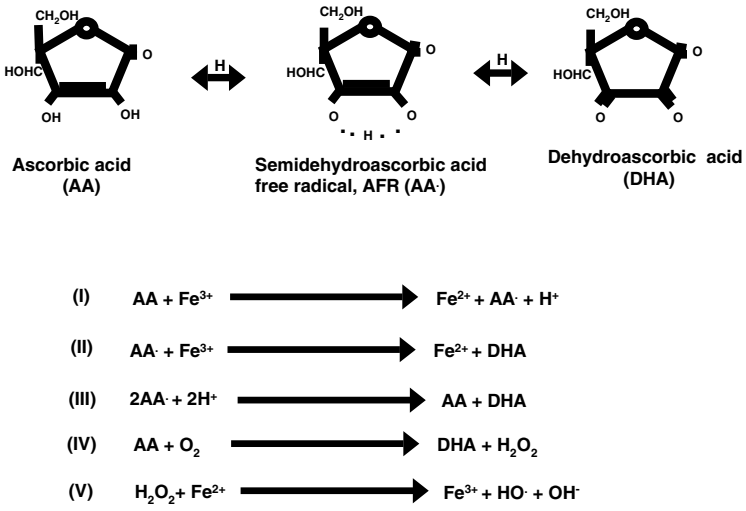

FIG. 14. Scheme of mechanisms of autoxidation of vitamin C in the presence of iron and generation of AFR and ROS. Vitamin C (ascorbic acid, AA) undergoes autoxidation in the presence of trace metals (iron) in two successive steps of one-electron oxidation, resulting in the formation of AFR (AA•) and dehydroascorbic acid (DHA) (2). In the presence of iron, AA acts as a potent prooxidant upon reducing Fe^{3+} to Fe^{2+} and is converted to AA• (I) (68). The AFR (AA•) may either reduce a Fe^{3+} species to Fe^{2+} form (II) or be converted to AA and DHA (III). H_2O_2 is also generated during the autoxidation of AA in the presence of O_2 (IV), which subsequently undergoes the Fenton reaction to form the highly reactive HO• radicals (V).

that vitamin C, at 1 m*M* concentration, induces generation of H_2O_2 in two different types of tissue culture media, Dulbecco's modified Eagle's medium and RPMI 1640 (21). These studies by others support our current findings that vitamin C at pharmacological doses acted as prooxidant, generated oxidants, and induced oxidative stress in BLMVECs.

Vitamin C-induced morphological alterations have been observed in the rabbit corneal and bovine aortic ECs (67, 77). Vitamin C has been shown to induce morphological changes in the human prostatic carcinoma cells (DU145), including membranous blebs and aberrant microvillar morphology (36). Morphological changes induced by H_2O_2 in bovine pulmonary artery ECs (BPAECs) and human umbilical vein ECs have been documented (11, 31). Earlier, we have shown that oxidants, including H_2O_2 and diperoxovanadate, cause cytoskeletal remodeling in BPAECs (75, 76). The cytoskeletal

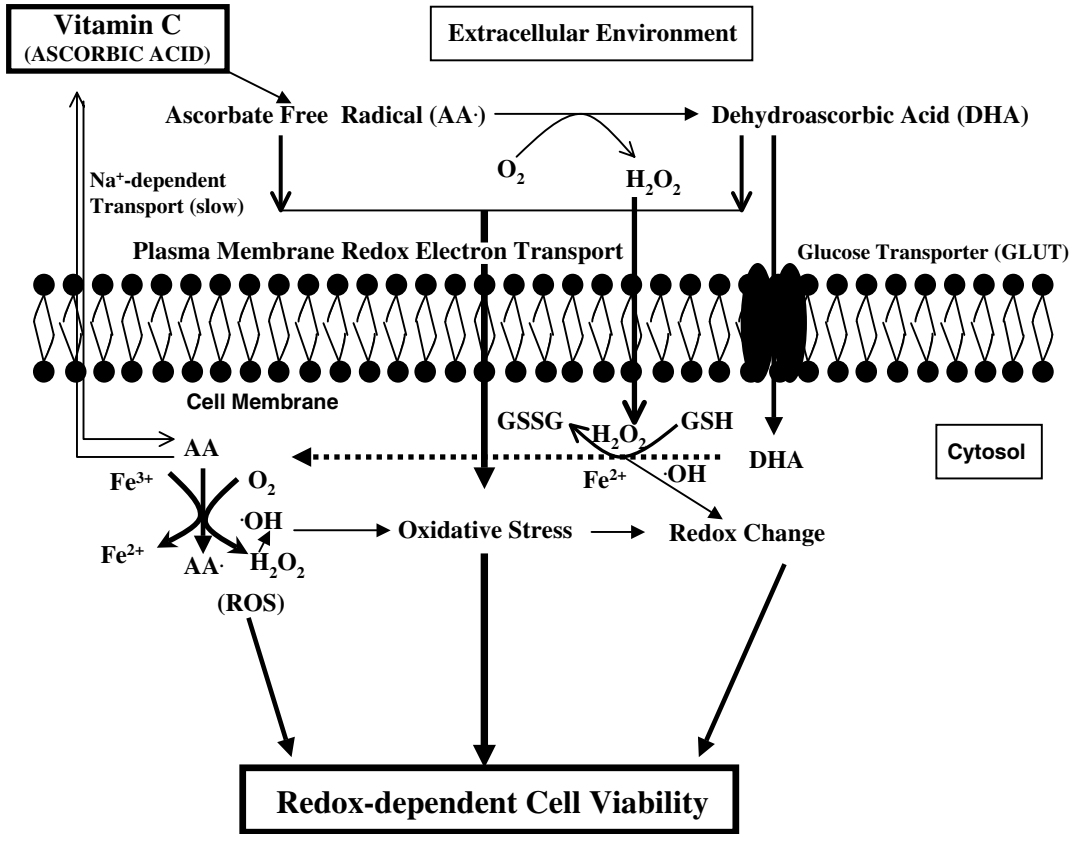

FIG. 15. Scheme of the proposed mechanism of vitamin C-induced oxidative stress and loss of redox-dependent cell viability in the lung microvascular ECs.

changes may very well be responsible for the oxidant-induced morphological changes in the cells. These studies support our current findings that vitamin C at pharmacological doses induced the alterations in cell morphology in BLMVECs wherein oxidative stress might have been involved. The precise mechanisms underlying the morphological alterations induced by vitamin C in BLMVECs are currently under investigation in our laboratory.

Oxidative stress-mediated cytotoxicity of vitamin C has been shown in different cultured cell systems (18, 20, 61). The vitamin C-induced cytotoxicity has been observed in the bovine aortic ECs (30). Antioxidants, including propyl gallate, have been shown to offer protection against oxidative stress and associated cytotoxicity in ECs and other cells (37, 58, 73). It has been established that oxidative stress causes a decrease or loss of the cellular thiol (GSH) pools (25). A dose-dependent depletion of the GSH levels in L929 fibroblast cells has been observed following exposure to vitamin C (66). The thiol protectants and replenishing agents such as cysteine, NAC, and GSH have been shown to reduce the radical intensity and cytotoxicity of ascorbate (65). It has been also reported that NAC protects against the vitamin C-induced loss of cell viability in the bovine aortic ECs (30). Earlier, we have also shown that oxidants including diamide, diperoxovanadate, and H_2O_2 deplete cellular thiols (GSH) in BLMVECs and BPAECs, which can be replenished by NAC (51, 75). These reports support our current findings that vitamin C caused alterations in the thiol redox leading to the observed loss of cell viability in BLMVECs.

The role of iron and other transition metals in oxidant-mediated injury and oxidative stress has been established (15). It has been shown that iron and other transition metals enhance the AFR intensity and cytotoxicity of vitamin C in human glioblastoma T98G cells (65). Vitamin C, at pharmacological doses, has been shown to enhance the release of iron from ferritin in neuroblastoma cells, which acts as a Fenton agent in propagating the cytotoxicity of vitamin C (3). It has been established that vitamin C reacts with transition metals resulting in the formation of H_2O_2, which subsequently undergoes Fenton reaction leading to the generation of the highly reactive $^{\bullet}OH$ (Fig. 14) (22). Desferal inhibition of ROS-induced cytotoxicity and the role of cellular stored iron have been shown in bovine aortic ECs (33). Our findings are in agreement with those of others and suggest the participation of intracellular iron in the vitamin C-induced loss of redox-dependent cell viability in BLMVECs.

Reports have been made on the cytotoxicity of H_2O_2 to porcine pulmonary artery ECs, BPAECs, and bovine carotid artery ECs (9, 19, 40). Cytotoxicity of vitamin C to the fibroblasts has been shown to be due to the formation of H_2O_2 (53). Catalase has been shown to attenuate the vitamin C-induced cytotoxicity in the human squamous carcinoma HSC-4 cells (63). It has been also suggested that vitamin C plays a dual role in both the generation of H_2O_2 and maintenance of iron in the Fe^{2+} state for efficient catalysis of the Fenton reaction (56). Cell death in the U937 cells induced by vitamin C at pharmacological doses is protected by the addition of catalase or the intracellular iron chelator, *o*-phenanthroline, to the medium, suggesting that H_2O_2 is solely responsible for the vitamin C-induced cell killing (69). Catalase when present in the culture medium also has been shown to protect against the vitamin C-induced reduction of cell viability in the bovine aortic ECs, further suggesting the role of H_2O_2 in the vitamin C-induced cytotoxicity (30). These studies support our current findings that vitamin C-induced loss of redox-dependent cell viability in BLMVECs was mediated by oxidative stress wherein H_2O_2 was crucially involved in the generation of the toxic $^{\bullet}OH$ species by the iron-mediated Fenton reaction.

The pharmacokinetic behavior of vitamin C in humans pertinent to its absorption, concentrations in blood plasma, and levels in urine has been thoroughly investigated and established (47). The concentrations of vitamin C in the plasma of unsupplemented humans are between 30 and 60 μ*M* and may double upon oral supplementation (42). Upon oral consumption of vitamin C, the plasma concentrations are tightly controlled and excess vitamin C is excreted (48). At a daily dose of 400 mg of oral vitamin C supplementation in humans, plasma is saturated attaining a steady-state concentration of 80 μ*M* of the vitamin (48). Unlike the tight control of plasma concentrations of vitamin C following oral supplementation, intravenous administration bypasses that tight control and results in high plasma (30–70-fold) and urine concentrations of vitamin C (48). Pharmacokinetic modeling shows that intravenous infusion of 50 g of vitamin C in healthy men can raise the plasma concentrations of vitamin C up to 13,400 μmol/L (49). It has been shown that ECs and leukocytes accumulate ~3–8 m*M* vitamin C (8, 24). Although the role of vitamin C in the EC function and integrity is not known in detail, it has been advocated that high concentrations of vitamin C accumulated in the ECs may offer protection against oxidative stress. The paradoxical nature of the dual-acting vitamin C may very well force the vitamin to act as a prooxidant at pharmacological doses in the physiological iron-containing environments, thus causing oxidative stress and toxicity. Based on the findings of the present study, it is concluded that vitamin C, at pharmacological concentrations, induces oxidative stress in the lung microvascular ECs by generating ROS wherein H_2O_2 and intracellular iron play a critical role, thus leading to depletion of GSH and loss of redox-dependent cell viability (Fig. 15). Although the modest vasodilatory effects are noticed at supraphysiological plasma concentrations of vitamin C, the clinical significance of these findings is unclear (48). The antioxidant and prooxidant actions of vitamin C at pharmacological doses in the vascular endothelium, upon infusion *in vivo*, warrant a thorough investigation for the use of this antioxidant in therapy of cancers and vascular disorders.

ACKNOWLEDGMENTS

This work was supported by grants from the National Heart, Lung, and Blood Institutes (HL69909, PO1 HL58064, HL38324, HL63744, and HL65608) and the American Lung Association.

ABBREVIATIONS

AFR, ascorbate free radical; BLMVEC, bovine lung microvascular endothelial cell; BPAEC, bovine pulmonary artery endothelial cell; DCFDA, 6-carboxy-2′,7′-dichlorodi-

hydroxyfluorescein diacetate dicarboxy methyl ester; DMPO, 5,5-dimethy-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; EC, endothelial cell; EPR, electron paramagnetic resonance; FBS, fetal bovine serum; GLUT, glucose transporter; GSH, γ-glutamylcysteinylglycine, glutathione; H_2O_2, hydrogen peroxide; MEM, minimal essential medium; MnTBAP, manganese(III) tetrakis(4-benzoic acid)porphyrin; MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; NAC, *N*-acetylcysteine; $O_2^{-\bullet}$, superoxide anion radical; $^{\bullet}OH$, hydroxyl radical; PBS, phosphate-buffered saline; ROS, reactive oxygen species, SOD, superoxide dismutase; SVCT, sodium-dependent vitamin C transporter.

REFERENCES

1. Anderson M and Grankvist K. Ascorbate-induced free radical toxicity to isolated islet cells. *Int J Biochem* 27: 493–498, 1995.
2. Arrigoni O and De Tullio MC. Ascorbic acid: much more than just an antioxidant. *Biochim Biophys Acta* 1569: 1–9, 2002.
3. Badder SL, Bruchelt G, Carmine TG, Lode HN, Rieth AG, and Niethammer, D. Ascorbic acid-mediated iron release from cellular ferritin and its relation to the formation of DNA strand breaks in neuroblastoma cells. *J Cancer Res Clin Oncol* 120: 415–421, 1994.
4. Balla J, Vercellotti GM, Nath K, Yachie A, Nagy E, Eaton JW, and Balla G. Haem, haem oxygenase and ferritin in vascular endothelial cell injury. *Nephrol Dial Transplant* 18: v8–v12, 2003.
5. Barbera JA, Peinado VI, and Santos S. Pulmonary hypertension in chronic obstructive pulmonary disease. *Eur Respir J* 21: 892–905, 2003.
6. Beckman JA, Goldfine AB, Gordon MB, and Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* 103: 1618–1623, 2001.
7. Bellomo G and Mirabelli F. Oxidative stress and cytoskeletal alterations. *Ann N Y Acad Sci* 663: 97–109, 1992.
8. Bergsten P, Amitai G, Kehr J, Dhariwal KR, Kelin HG, and Levine M. Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes. Depletion and reaccumulation. *J Biol Chem* 265: 2584–2587, 1990.
9. Block ER. Hydrogen peroxide alters the physical state and function of the plasma membrane of pulmonary artery endothelial cells. *J Cell Physiol* 146: 362–369, 1991.
10. Bors W and Buettner GR. The vitamin C radical and its reactions. In: *Vitamin C in Health and Disease*, edited by Packer L and Fuchs J. New York, NY: Marcel Dekker, 1997, pp. 75–94.
11. Bradley JR, Thiru S, and Pober JS. Hydrogen peroxide-induced endothelial retraction is accompanied by a loss of the normal spatial organization of endothelial cell adhesion molecules. *Am J Pathol* 147: 627–641, 1995.
12. Bray TM. Antioxidants and oxidative stress in health and disease: Introduction. *Proc Soc Exp Biol Med* 222: 195, 1999.
13. Buettner GR and Jurkiewicz BA. Ascorbate free radical as a marker of oxidative stress: an EPR study. *Free Radic Biol Med* 14: 49–55, 1993.
14. Buettner GR and Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 145: 532–541, 1996.
15. Cai L, Koropatnick J, and Cherian MG. Roles of vitamin C in radiation-induced DNA damage in presence and absence of copper. *Chem Biol Interact* 137: 75–88, 2001.
16. Carr AC and Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. *Am J Clin Nutr* 69: 1086–1107, 1999.
17. Carr A and Frei B. The role of natural antioxidants in preserving the biological activity of endothelium-derived nitric oxide. *Free Radic Biol Med* 28: 1806–1814, 2000.
18. Casciari JJ, Riordan NH, Schmidt TL, Meng XL, Jackson JA, and Riordan HD. Cytotoxicity of ascorbate, lipoic acid, and other antioxidants in hollow fibre in vitro tumours. *Br J Cancer* 84: 1544–1550, 2001.
19. Chen SH, Wu HL, Lin MT, Jen CJ, Wing LY, Tsao CJ, and Chang WC. Cytoprotective effect of reduced glutathione in hydrogen peroxide-induced endothelial injury. *Prostaglandins Leukot Essent Fatty Acids* 45: 299–305, 1992.
20. Chepada T, Cadau M, Girin P, Frey J, and Chamson A. Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. *In Vitro Cell Dev Biol Anim* 37: 26–30, 2001.
21. Clement MV, Ramalingam J, Long LH, and Halliwell B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid Redox Signal* 3: 157–163, 2001.
22. Deutsch JC. Ascorbic acid oxidation by hydrogen peroxide. *Anal Biochem* 255: 1–7, 1998.
23. Dröge W. Oxidative stress and aging. *Adv Exp Med Biol* 543: 191–200, 2003.
24. Ek A, Strom K, and Cotgreve IA. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. *Biochem Pharmacol* 50: 1339–1346, 1995.
25. Forman HJ, Dickinson DA, and Iles KE. HNE-signaling pathways leading to its elimination. *Mol Aspects Med* 24: 189–194, 2003.
26. Grossmann M, Dobrev D, Himmel HM, Ravens U, and Kirch W. Ascorbic acid-induced modulation of venous tone in humans. *Hypertension* 37: 949–954, 2001.
27. Halliwell B. Vitamin C: antioxidant or pro-oxidant in vivo? *Free Radic Res* 25: 439–454, 1996.
28. Halliwell B. Vitamin C: poison, prophylactic or panacea? *Trends Biochem Sci* 24: 255–259, 1999.
29. Hamabe A, Takase B, Uehata A, Kurita A, Ohsuzu F, and Tamai S. Impaired endothelium-dependent vasodilation in brachial artery in variant angina pectoris and the effect of intravenous administration of vitamin C. *Am J Cardiol* 87: 1154–1159, 2001.
30. Hanashima C and Namiki H. Reduced viability of vascular endothelial cells by high concentration of ascorbic acid in vitreous humor. *Cell Biol Int* 23: 287–298, 1999.
31. Hinshaw DB, Burger JM, Armstrong BC, and Hyslop, PA. Mechanisms of endothelial shape change in oxidant injury. *J Surg Res* 46: 339–349, 1989.
32. Hirai N, Kawano H, Hirashima O, Motoyama T, Moriyama Y, Sakamoto T, Kugiyama K, Ogawa H, Nakao K, and Yasue H. Insulin resistance and endothelial dysfunction in

smokers: effects of vitamin C. *Am J Physiol Heart Circ Physiol* 279: H1172–H1178, 2000.
33. Hiraishi H, Terano A, Razandi M, Pedram A, Sugimoto T, Harada T, and Ivey KJ. Reactive oxygen metabolite-induced toxicity to cultured bovine endothelial cells: status of cellular iron in mediating injury. *J Cell Physiol* 160: 132–134, 1994.
34. Hirashima O, Kawano H, Motoyama T, Nobutaka H, Ohagushi M, Kugiyama K, Ogawa H, and Yasue H. Improvement of endothelial function and insulin sensitivity with vitamin C in patients with coronary spastic angina. *Am J Cardiol* 35: 1860–1866, 2000.
35. Hoey J. Editorial: a cure for miracles. *Can Med Assoc J* 164: 317, 2001.
36. Jamison M, Gilloteaux J, Venugopal M, Koch JA, Sowick C, Shah R, and Summers JL. Flow cytometric and ultrastructural aspects of the synergistic antitumor activity of vitamin C and vitamin K3 combinations against human prostatic carcinoma cells. *Tissue Cell* 28: 687–701, 1996.
37. Kahl R and Hildebrandt AG. Methodology for studying antioxidant activity and mechanisms of action of antioxidants. *Food Chem Toxicol* 24: 1007–1014, 1986.
38. Kruszewski M. Labile iron pool: the main determinant of cellular response to oxidative stress. *Mutat Res* 531: 81–92, 2003.
39. Langlois M, Duprez D, Delanghe J, De Buyzere M, and Clement DL. Serum vitamin C concentration is low in peripheral arterial disease and is associated with inflammation and severity of atherosclerosis. *Circulation* 103: 1863–1868, 2001.
40. Li L and Lau BH. A simplified in vitro model of oxidant injury using vascular endothelial cells. *In Vitro Cell Dev Biol Anim* 29: 531–536, 1993.
41. May JM. Ascorbate function and metabolism in the human erythrocyte. *Front Biosci* 3: 1–10, 1998.
42. May JM. How does ascorbic acid prevent endothelial dysfunction? *Free Radic Biol Med* 28: 1421–1429, 2000.
43. May JM, Qu Z, and Li X. Requirement for GSH in recycling of ascorbic acid in endothelial cells. *Biochem Pharmacol* 62: 873–881, 2001.
44. Middleton E Jr, Kandaswami C, and Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 52: 673–751, 2000.
45. Natarajan V and Garcia JGN. Agonist-induced activation of phospholipase D in bovine pulmonary artery endothelial cells: regulation by protein kinase C and calcium. *J Lab Clin Med* 121: 337–347, 1993.
46. Padayatty JS and Levine M. Reevaluation of ascorbate in cancer treatment: emerging evidence, open minds and serendipity. *J Am Coll Nutr* 19: 423–425, 2000.
47. Padayatty JS and Levine M. New insights into physiology and pharmacology of vitamin C. *Can Med Assoc J* 164: 353–355, 2001.
48. Padayatty JS, Katz A, Wang Y, Eck P, Kwon O, Lee J-H, Chen S, Corpe C, Dutta A, Dutta SK, and Levine M. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr* 22: 18–35, 2003.
49. Padayatty JS, Sun H, Wang Y, Riordan HD, Hewitt SM, Katz A, Wesley RA, and Levine M. Vitamin C pharmacokinetics: implications for oral and intravenous use. *Ann Intern Med* 140: 533–537, 2004.
50. Paolini M, Pozzetti L, Pedulli GF, Marchesi E, and Cantelli-Forti G. The nature of prooxidant activity of vitamin C. *Life Sci* 64: 273–278, 1999.
51. Parinandi NL, Scribner WM, Vepa S, Shi S, and Natarajan V. Phospholipase D activation in endothelial cells is redox sensitive. *Antioxid Redox Signal* 1: 193–210, 1999.
52. Parinandi NL, Kleinberg MA, Usatyuk PV, Cummings RJ, Pennathur A, Cardounel AJ, Zweier, JL, Garcia JGN, and Natarajan V. Hyperoxia-induced NAD(P)H oxidase activation and regulation by MAP kinases in human lung endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 284: L26–L38, 2003.
53. Peterkofsky B and Prather W. Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. *J Cell Physiol* 90: 61–70, 1997.
54. Pietri S, Culcasi M, Stella L, and Cozzone PJ. Ascorbyl free radical as a reliable indicator of free-radical-mediated myocardial ischemic and post-ischemic injury. A real-time continuous flow ESR study. *Eur J Biochem* 193: 845–854, 1990.
55. Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, and Lunec J. Vitamin C exhibits pro-oxidant properties. *Nature* 392: 559, 1998.
56. Prat AG and Turrens JF. Ascorbate- and hemoglobin-dependent brain chemiluminescence. *Free Radic Biol Med* 8: 319–325, 1990.
57. Roomi MW, House D, Eckert-Maksic M, Maksic ZB, and Tsao CS. Growth suppression of malignant leukemia cell line in vitro by ascorbic acid (vitamin C) and its derivatives. *Cancer Lett* 122: 93–99, 1998.
58. Roy S, Parinandi N, Zeigelstein R, Hu Q, Pei Y, Travers JB, and Natarajan V. Hyperoxia alters phorbol ester-induced phospholipase D activation in bovine lung microvascular endothelial cells. *Antioxid Redox Signal* 5: 217–228, 2003.
59. Sakagami H and Satoh K. Effect of ascorbate oxidase on radical intensity and cytotoxic activity of ascorbate. *Anticancer Res* 17: 1163–1166, 1997.
60. Sakagami H and Satoh K. Modulating factors of radical intensity and cytotoxic activity of ascorbate (review). *Anticancer Res* 17: 3513–3520, 1997.
61. Sakagami H and Satoh K. Detection of drug–drug interaction by ESR spectroscopy. *Nippon Yakurigaku Zasshi* 113: 349–356, 1999.
62. Sakagami H, Satoh K, Ohata H, Takahashi H, Yoshida H, Iida M, Kuribayashi N, Sakagami T, Momose K, and Takeda M. Relationship between ascorbyl radical intensity and apoptosis-inducing activity. *Anticancer Res* 16: 2635–2644, 1996.
63. Sakagami H, Satoh K, Ida Y, Hosaka M, Arakawa H, and Maeda M. Interaction between sodium ascorbate and dopamine. *Free Radic Biol Med* 25: 1013–1020, 1998.
64. Salacinski HJ, Punshon G, Krijgsman B, Hamilton G, and Seifalian AM. A hybrid compliant vascular graft seeded with microvascular endothelial cells extracted from human omentum. *J Artif Organs* 25: 974–982, 2001.
65. Satoh K and Sakagami H. Effect of metal ions on radical intensity and cytotoxic activity of ascorbate. *Anticancer Res* 17: 1125–1129, 1997.

66. Schmidt RJ, Chung LY, Andrews AM, and Turner TD. Toxicity of L-ascorbic acid to L929 fibroblast cultures: relevance to biocompatibility testing of materials for use in wound management. *J Biomed Mater Res* 27: 521–530, 1993.
67. Schor AM, Schor SL, and Allen TD. Effects of culture conditions on the proliferation, morphology and migration of bovine aortic endothelial cells. *J Cell Sci* 62: 267–285, 1983.
68. Schrammel A, Koesling D, Schmidt K, and Mayer B. Inhibition of purified soluble guanylyl cyclase by L-ascorbic acid. *Cardiovascular Res* 47: 602–608, 2000.
69. Sestili P, Brandi G, Brambilla L, Cattabeni F, and Cantoni O. Hydrogen peroxide mediates the killing of U937 tumor cells elicited by pharmacologically attainable concentrations of ascorbic acid: cell death prevention by extracellular catalase or catalase from cocultured erythrocytes or fibroblasts. *J Pharmacol Exp Ther* 277: 1719–1725, 1996.
70. Sherman DL, Keaney JF, Biegelsen ES, Duffy SJ, Coffman JD, and Vita JA. Pharmacological concentrations of ascorbic acid are required for the beneficial effect on endothelial vasomotor function in hypertension. *Hypertension* 35: 936–941, 2000.
71. Song JH, Shin SH, and Ross GM. Prooxidant effects of ascorbate in rat brain slices. *J Neurosci Res* 58: 328–336, 1999.
72. Tiwari A, Salacinski HJ, Punshon G, Hamilton G, and Seifalian AM. Development of a hybrid cardiovascular graft using a tissue engineering approach. *FASEB J* 16: 791–796, 2002.
73. Toborek M, Blanc EM, Kaiser S, Mattson MP, and Hennig B. Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured vascular endothelial cells. *J Lipid Res* 38: 2155–2167, 1997.
74. Usatyuk PV, Fomin VP, Shi S, Garcia JG, Schaphorst K, and Natarajan V. Role of Ca^{2+} in diperoxovanadate-induced cytoskeletal remodeling and endothelial cell barrier function. *Am J Physiol Lung Cell Mol Physiol* 285: L1006–L1007, 2003.
75. Usatyuk PV, Vepa S, Watkins T, He D, Parinandi NL, and Natarajan V. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid Redox Signal* 5: 723–730, 2003.
76. Vepa S, Scribner WM, Parinandi NL, English D, Garcia JG, and Natarajan V. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. *Am J Physiol* 277: L150–L158, 1999.
77. Yue BY, Niedra R, and Baum JL. Effects of ascorbic acid on cultured corneal endothelial cells. *Invest Ophthalmol Vis Sci* 19: 1471–1476, 1980.
78. Zweier JL, Broderick R, Kuppusamy P, Thompson-Gorman S, and Lutty GA. Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J Biol Chem* 269: 24156–24162, 1994.

Address reprint requests to:
Narasimham L. Parinandi, Ph.D.
Dorothy M. Davis Heart and Lung Research Institute
Room 611-A, 473 West 12th Avenue
The Ohio State University
Columbus, OH 43210-1252

E-mail: Parinandi-1@medctr.osu.edu

Received for publication June 30, 2004; accepted August 23, 2004.

This article has been cited by:

1. Corey J. Miller, Sellamuthu S. Gounder, Sankaranarayanan Kannan, Karan Goutam, Vasanthi R. Muthusamy, Matthew A. Firpo, J. David Symons, Robert Paine, John R. Hoidal, Namakkal Soorappan Rajasekaran. 2012. Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1822**:6, 1038-1050. [CrossRef]
2. Andreas Bracher , Stephen F. Doran , Giuseppe L. Squadrito , Edward M. Postlethwait , Larry Bowen , Sadis Matalon . Targeted Aerosolized Delivery of Ascorbate in the Lungs of Chlorine-Exposed Rats. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
3. Nizar Abd Manan, Norazlina Mohamed, Ahmad Nazrun Shuid. 2012. Effects of Low-Dose versus High-Dose #-Tocotrienol on the Bone Cells Exposed to the Hydrogen Peroxide-Induced Oxidative Stress and Apoptosis. *Evidence-Based Complementary and Alternative Medicine* **2012**, 1-10. [CrossRef]
4. Seung-Ah Lee, Young-Ok Son, Sung-Ho Kook, Ki-Choon Choi, Jeong-Chae Lee. 2011. Ascorbic acid increases the activity and synthesis of tyrosinase in B16F10 cells through activation of p38 mitogen-activated protein kinase. *Archives of Dermatological Research* . [CrossRef]
5. Namakkal Soorappan Rajasekaran , Saradhadevi Varadharaj , Gayatri D. Khanderao , Christopher J. Davidson , Sankaranarayanan Kannan , Matthew A. Firpo , Jay L. Zweier , Ivor J. Benjamin . 2011. Sustained Activation of Nuclear Erythroid 2-Related Factor 2/Antioxidant Response Element Signaling Promotes Reductive Stress in the Human Mutant Protein Aggregation Cardiomyopathy in Mice. *Antioxidants & Redox Signaling* **14**:6, 957-971. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
6. Shang-U Kim, Yangping Liu, Kevin M. Nash, Jay L. Zweier, Antal Rockenbauer, Frederick A. Villamena. 2010. Fast Reactivity of a Cyclic Nitrone-Calix[4]pyrrole Conjugate with Superoxide Radical Anion: Theoretical and Experimental Studies †. *Journal of the American Chemical Society* **132**:48, 17157-17173. [CrossRef]
7. Silvia Nelli, John Craig, William Martin. 2009. Oxidation by trace Cu2+ ions underlies the ability of ascorbate to induce vascular dysfunction in the rat perfused mesentery. *European Journal of Pharmacology* **614**:1-3, 84-90. [CrossRef]
8. Emily Steinhour, Shariq I. Sherwani, Jessica N. Mazerik, Valorie Ciapala, Elizabeth O'Connor Butler, Jason P. Cruff, Ulysses Magalang, Sampath Parthasarathy, Chandan K. Sen, Clay B. Marsh, Periannan Kuppusamy, Narasimham L. Parinandi. 2008. Redox-active antioxidant modulation of lipid signaling in vascular endothelial cells: vitamin C induces activation of phospholipase D through phospholipase A2, lipoxygenase, and cyclooxygenase. *Molecular and Cellular Biochemistry* **315**:1-2, 97-112. [CrossRef]
9. A FATOKUN, T STONE, R SMITH. 2008. Responses of differentiated MC3T3-E1 osteoblast-like cells to reactive oxygen species. *European Journal of Pharmacology* **587**:1-3, 35-41. [CrossRef]
10. Narasimham L. Parinandi , Ashish Sharma , Timothy D. Eubank , Bruce F. Kaufman , Vijay Kumar Kutala , Clay B. Marsh , Louis J. Ignarro , Periannan Kuppusamy . 2007. Nitroaspirin (NCX-4016), an NO Donor, is Antiangiogenic Through Induction of Loss of Redox-Dependent Viability and Cytoskeletal Reorganization in Endothelial Cells. *Antioxidants & Redox Signaling* **9**:11, 1837-1850. [Abstract] [Full Text PDF] [Full Text PDF with Links]
11. H OHTA, I OKAMOTO, T HANAYA, S ARAI, T OHTA, S FUKUDA. 2006. Enhanced antioxidant defense due to extracellular catalase activity in Syrian hamster during arousal from hibernation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **143**:4, 484-491. [CrossRef]