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Enhanced Protection Against Peroxidation-Induced Mortality of Aortic Endothelial Cells by Ascorbic Acid-2-O-Phosphate Abundantly Accumulated in the Cell as the Dephosphorylated Form

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Bovine aortic endothelial BAE-2 cells exposed to the peroxidizing agent, tert-butylhydroperoxide (t-BuOOH) or 2,4-nonadienal (NDE), suffered from disruption of cell membrane integrity and from reduction of mitochondrial dehydrogenase activity as assessed by fluorometry using ethidium homodimer and photometry using WST-1, respectively. The cells were protected from t-BuOOH-induced injury more markedly by L-ascorbic acid-2-O-phosphate (Asc2P) stably masked at the 2,3-enediol moiety, which is responsible for the antioxidant ability of L-ascorbic acid (Asc), than by Asc itself. In contrast, NDE-induced membrane disruption but not mitochondrial dysfunction was prevented by Asc2P, whereas Asc exhibited no prevention against both types of injury. The amount of intracellular Asc was 7.2- to 9.0-fold larger in Asc2P-administered BAE-2 cells, where the intact form Asc2P was not detected, than in Asc-administered cells as assessed by HPLC of cell extract with detection by coulometric ECD and UV. During transmembrane influx into the cell, Asc2P was concentrated as highly as 70- to 90-fold relative to the extracellular Asc2P concentration, whereas Asc was 8- to 13-fold concentrated as estimated based on an intracellular water content of 0.59 pL/cell determined by [^{14}C]PEG/gas chromatography. Thus, Asc2P but not Asc is highly concentrated in the aqueous phase of the cell after prompt dephosphorylation, and may thereby render the cell more resistant to t-BuOOH-peroxidation assumedly via scavenging of intracellular reactive oxygen species than to peroxidation with the less hydrophilic agent NDE.

Keywords: Ascorbic acid-2-O-phosphate, Peroxidizing agent, Cell Mortality, Ascorbate transport, Cell membrane integrity disruption, Endothelial cells

INTRODUCTION

L-Ascorbic acid (Asc) is known to be so labile to oxidation in aqueous solution as to be irreversibly decomposed via dehydroascorbic acid. As a long-lasting Asc form masked at the 2,3-enediol moiety responsible for antioxidant ability,

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Asc-2-O-phosphate (Asc2P) and Asc-2-O-alpha-glucoside exert diverse pharmacological actions such as promotion of the organization of extracellular matrix in fibroblasts,[1,2] enhanced activity of alkaline phosphatase in osteoblasts,[3] accelerated differentiation of adipocytes[4] and enhanced antibody production by splenocytes,[5] any of which have not been observed markedly for the parent form Asc. However, the Asc derivative has scarcely been analyzed for transport mechanism across the cell membrane.[6]

Asc can be cytotoxic under some circumstances: chelation of one ferrous ion per the molecule of Asc is carcinostatic through marked retention of redox half-life during cell contact than that of Asc.[7] Cytotoxicity is acquired by Asc derivatives monoacylated at the C6-hydroxyl site with palmitoyl groups, but not di- or triacylated,[8] which generate some reactive oxygen species (ROS) other than hydrogen peroxide at earlier stages,[9] induce marked release of membrane phospholipids into the extracellular space,[10] and post-transcriptionally inhibit ornithine decarboxylase activity resulting in inhibition of polyamine synthesis.[11] Furthermore, Asc itself inhibits DNA synthesis and proliferation of cells grown at lower cell densities.[12] Thus, cytotoxicity can be exhibited under specified conditions by Asc which contains an intact 2,3-enediol moiety, whereas an Asc derivative blocked at the C2-hydroxyl group with glucoside or phosphate is not cytotoxic to even sparsely cultured cells,[13] but prevents hydrocortisone-induced cataract formation by inhibition of lipid peroxidation.[14] However, the mechanism whereby cytotoxicity is not exhibited by the Asc derivative remains to be analyzed in terms of ROS generation or contents of intracellular Asc.

In the present study we have examined whether peroxidation-induced cell mortality was prevented more effectively by Asc2P than by Asc, and whether the preventive effects was correlated with the transmembrane influx of Asc2P or Asc into the cell.

MATERIALS AND METHODS

Cell Culture

Vascular endothelial cells BAE-2 (6×10^5 cells) obtained from bovine aorta were used at population doubling levels of 29–59. Cells seeded on a 60-mm dish were cultured in Eagle's minimum essential medium (MEM, Nissui Pharm., Tokyo) supplemented with 10% dialyzed and inactivated fetal bovine serum (FBS, Gibco). The subconfluent cells were treated for subculture with 0.25% trypsin-0.02% EDTA in PBS(–), neutralized with an equivoluminal MEM-10% FBS and detached with a scraper. The cell number was determined with a Coulter electric particle counter ZM.

Peroxidation Treatment

BAE-2 cells ($7–8 \times 10^4$ cells) in a logarithmic growth phase were seeded in a Sumilon or Corning 24-well microplate and were precultured for 24 hr. The medium was then replaced by fresh MEM-10% FBS containing Asc2P (Showa Denko, Tokyo) or Asc (Sigma) and was incubated for 22–24 hr. The cells were administered for 3 or 7 hr with tert-butyl-hydroperoxide (t-BuOOH) or 2,4-nonadienal (NDE) dissolved in 1 ml of 0.02% ethanol-Hanks' balanced salt solution (HBSS, Gibco) and concurrently with Asc/derivative. The control cells received HBSS containing no peroxidizing agent or Asc/derivative.

Fluorometry of a Dead Cell-Specific DNA-Strand Intercalator

Cells peroxidized or administered with Asc/derivative underwent aspiration of the medium and received HBSS of 1 ml containing 5 uM ethidium homodimer (Et_2, Millipore) for 30 min. Fluorescence of cellular DNA-bound Et_2 was determined with a Millipore microplate fluorometer Cytofluor 2350 (excitation: 485 nm; emission: 645 nm).

Microtiter Photometric Assay for Mitochondrial Dehydrogenage Activity

The treated or control cells underwent aspiration of the medium and received 300 ul of phenol red-free MEM-10% FBS and 30 ul of WST-1 solution (Wako, Osaka). After 3-hr incubation, the medium was transferred into a 96-well microplate and was measured with a Bio-Rad microplate photometer model 3550.

Ion Paired HPLC with Coulometric ECD and UV

After uptake of Asc, dehydroascorbic acid (DehAsc, Aldrich) or Asc2P for 22–24 hr, the cells were rinsed three times with MEM to remove adherent Asc/derivative thoroughly. Cells were then trypsinized, pelleted with a centrifuge and crushed with a pencil- or Potter-type homogenizer. After removal of proteinic ingredients with a Millipore filter Molcut II, the homogenate was injected on an octadecylsilica gel Shodex ODSpak F-411A column (Showa Denko, Tokyo; 4.6 × 150 mm) connected in a series circuit of Gilson HPLC system 305 and developed at 40°C and a flow rate of 1.5 ml/min. The mobile phase consisted of 0.1 M sodium acetate, 0.02% EDTA and 0.017 % n-octylamine. Asc/derivatives were detected with a coulometric electrochemical detector (ECD) Coulochem II (ESA, Bedford) at –200 mV/150 mV or with a Gilson UV detector 115 at 254/265 nm.

Intracellular Water Contents

An intracellular water content of BAE-2 cells was determined by [^{14}C]polyethylene glycol(PEG)/gas chromatographic method[15] with minor modification.[16] The cells of 2.84×10^7 were centrifuged, received [^{14}C]PEG of 6 uCi, weighed and extracted with methanol containing 4% butanol. Gas chromatography was performed with the use of a 10% PEG 6000 Shimalite F 3m stainless column built in a Shimazu gas chromatograph 4A with He gas carrier of 60 ml/min at 120°C. The cell samples were dried up, received Instagel (Packard) and were determined for the radioactivity with an Aloka liquid scintillation counter LC-3600.

Determination of Intracellular Asc Concentration

The cells were collected and counted with a Coulter electric particle counter ZM equipped with a channelyzer type 256. Based on values determined as intracellular water contents, intracellular Asc concentrations were calculated from Asc amounts determined by ion paired HPLC of cell extracts.

RESULTS

Disruption of the Cell Membrane Integrity by Peroxidizing Agents

Bovine aortic endothelial cells BAE-2 were treated with oxidant, t-BuOOH or NDE, and then were subjected to staining with the dead cell-specific DNA-strand intercalator Et_2, which can quantify disruption of the cell membrane integrity at an earlier stage of cell death than Trypan blue.[17] Et_2 stained densely the nuclei and faintly the cytoplasm of membrane-disrupted cells treated with t-BuOOH or NDE, but scarcely stained live cells as shown by comparison of fluorescent and normal microscopy for the same field. The fluorescence intensities of Et_2-stained cells, although unaltered between 1 and 5 μM of the oxidant, as observed for cell viability assay using WST-1 (not shown), progressively increased as a function of oxidant doses higher than 50 μM and the treatment period (Figure 1), suggesting that a correlation existed between the degree of membrane disruption and the fluorescence intensity attributed to Et_2 staining.

Protective Effects of Asc2P on Oxidant-induced Membrane Disruption

BAE-2 cells treated with t-BuOOH or NDE showed markedly increased fluorescence inten-

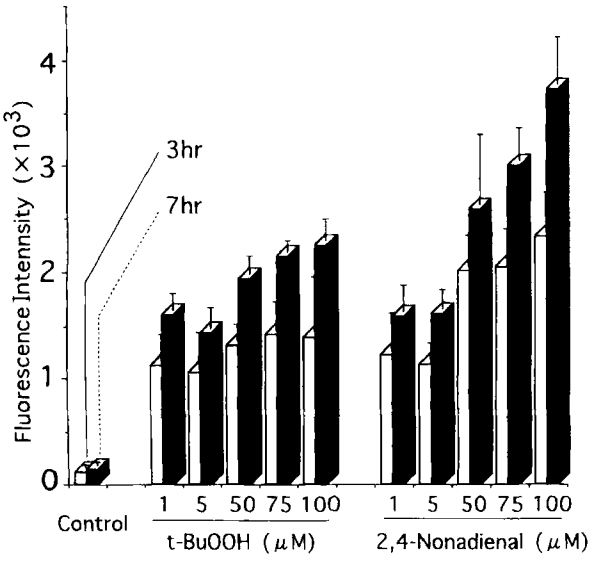


FIGURE 1 Effects of peroxidizing agents on disruption of the cell membrane integrity in bovine aortic endothelial BAE-2 cells. Cells in a 24-well microplate were treated with tert-butylhydroperoxide (t-BuOOH) or 2,4-nonadienal (NDE) in HBSS for 3 or 7 hr and subsequently replaced by fresh HBSS containing ethidium homodimer (Et_2). The fluorescence attributed to Et_2 bound to DNA strands in dead cells was measured with a microplate fluorometer. The control cells were treated with no oxidant. The data were typical of three independent experiments. The bar represents the S.D. of wells in triplicate. Note that, in contrast to no difference in fluorescence intensities between 1 and 5 μM of the peroxidants, dose-dependency was observed above 50 μM of both the oxidants.

sities attributed to Et_2, whereas the enhanced fluorescence was reduced more markedly by previous administration of Asc2P than of Asc (Fig. 2), suggesting that Asc2P protected more greatly the cells from oxidant-induced membrane disruption assumedly owing to superiority of Asc2P to Asc in terms of the ability to scavenge t-BuOOH- or NDE-derived ROS. No better protection was observed at the higher dose of Asc, or, except for t-BuOOH of 200 μM, Asc2P, which was more emphatically observed for cell viability assay using WST-1 (Fig. 4). The cytoprotection may be attributed to a portion of the administered Asc2P or Asc undergoing intracellular uptake, because it was observed when extracellular Asc2P or Asc was removed by rinsing before peroxidant treatment. The protection by Asc2P against oxidant-induced membrane disruption was also confirmed by fluorescence microscopy, where t-BuOOH treatment of the cells resulted in more extensive Et_2-staining in the absence than in the presence of Asc2P (Fig. 3).

Protection by Asc2P Against Oxidant-induced Viability Reduction

The cells exposed to t-BuOOH or NDE reduced the viability as assessed by microtiter photometry for mitochondrial dehydrogenage activity using a formazan dye-producing tetrazolium, WST-1 (Fig. 4).[29] The t-BuOOH-induced viabil-

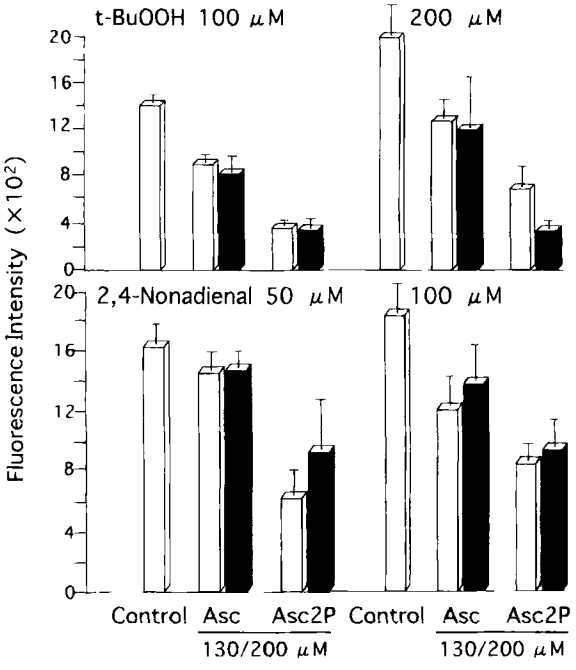


FIGURE 2 Protective effects of L-ascorbic acid-2-O-phosphate (Asc2P) on peroxidant-treated BAE-2 cells. Cells were previously administered with Asc2P or L-ascorbic acid (Asc) of 130 or 200 μM for 24 hr. The medium was then replaced by HBSS containing t-BuOOH or NDE of 50 or 100 μM and Asc/derivative of 130 or 200 μM, and was incubated for 7 hr. Cell mortality was determined by fluorometry using Et_2 as described in Figure 1. The fluorescence intensities were smaller as a whole than those of Figure 1 because the fluorometry was conducted at a fluorometric sensitivity of 6, which was lower than in Figure 1 (a sensitivity of 7) because of time-dependent drift of the baseline. The data were typical of four independent experiments. The bar represents the S.D. of wells in triplicate.

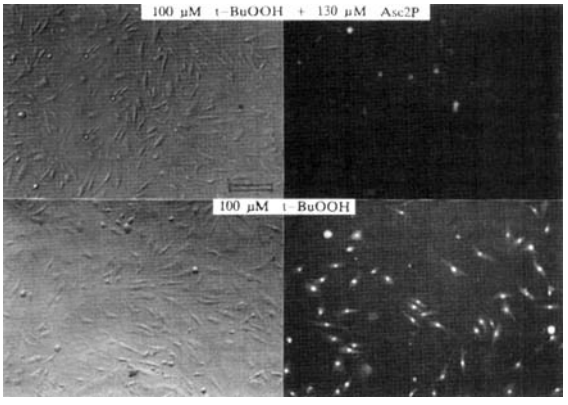


FIGURE 3 Fluorescence microphotographs demonstrating the cytoprotective effects of Asc2P against t-BuOOH-induced mortality of BAE-2 cells. Cells were exposed to t-BuOOH and/or Asc2P for 7 hr as described in Figure 2, and then fluorescence and normal microphotographs were taken. The scale indicates a length of 90 μm. (See Color Plate I at the back of this issue.)

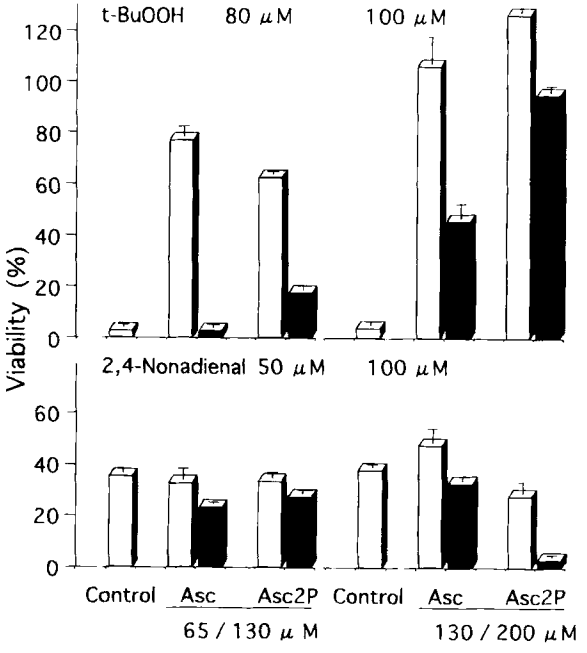
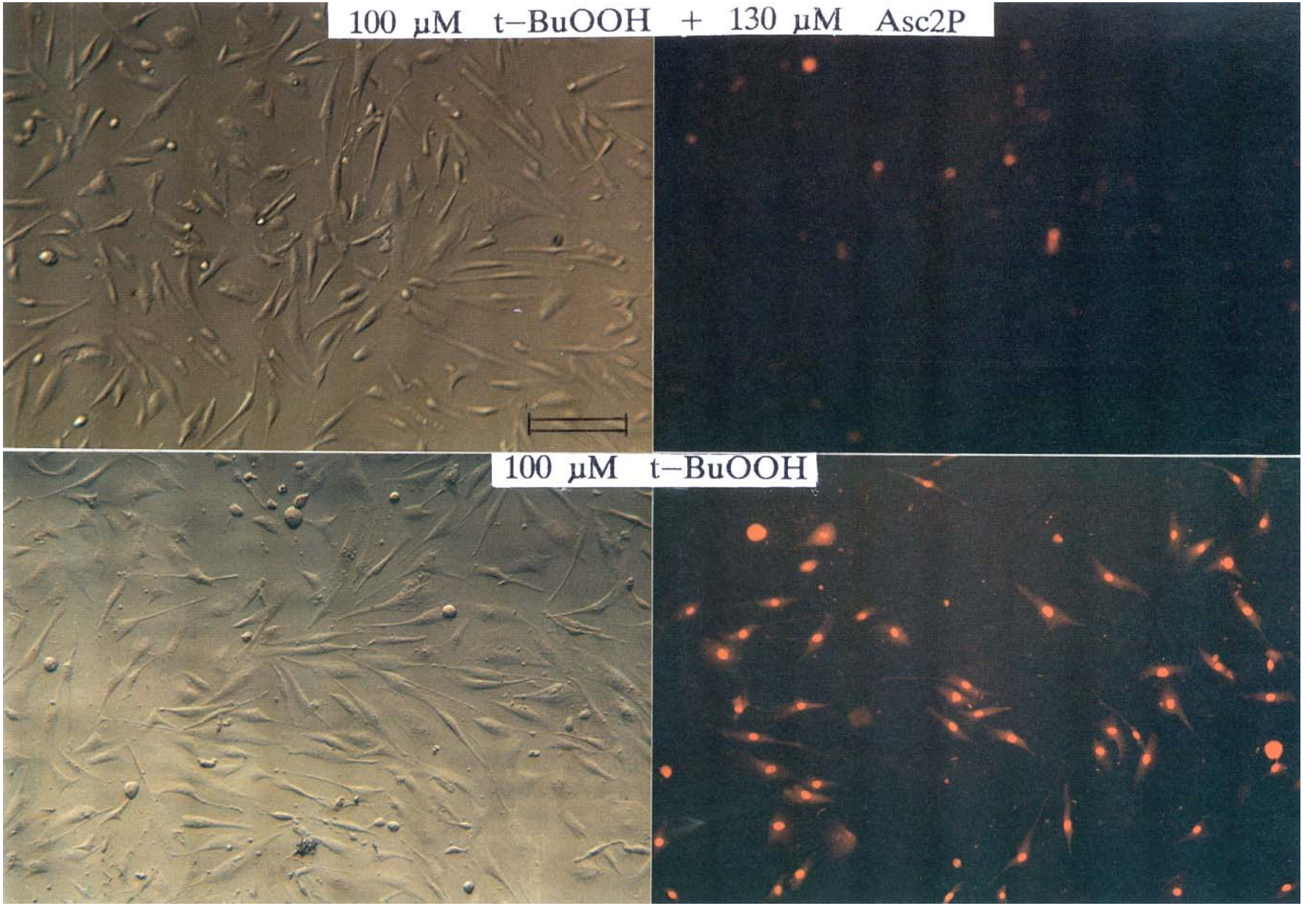


FIGURE 4 Preventive effects of Asc2P or Asc on peroxidant-induced reduction of viability of BAE-2 cells. Cells were previously administered with Asc2P or Asc, and treated with t-BuOOH or NDE as in Figure 2. After 24-hr culture, viability of the cells was determined by measurement of mitochondrial dehydrogenase activity with the formazan-producing tetrazolium WST-1. Viability of untreated cells is defined as 100%. Note that t-BuOOH-induced reduction of viability was prevented by Asc or Asc2P, the latter of which was more effective than the former at higher doses. The data were typical of three independent experiments. The bar represents the S.D. of wells in triplicate.

ity reduction was prevented by previous administration of Asc2P or Asc in the same manner as in Figure 2 except appreciable cytoprotection by Asc of lower doses. At higher doses, however, Asc2P prevented t-BuOOH-induced viability reduction more extensively for either 80 or 100 μM of t-BuOOH than Asc. In contrast, NDE, a more hydrophobic oxidant than t-BuOOH, induced less marked viability reduction, which was not prevented by Asc2P or Asc unlike Figure 2, suggesting the lower accessibility of hydrophilic Asc/derivative toward a deep part of the membrane phase of the cell assumed to be attacked by NDE.

Intracellular Asc Content in Asc2P-Administered Cells

To clarify the mechanism whereby Asc2P protected BAE-2 cells from the oxidant-induced cell injuries, the intracellular Asc content was determined for extracts from Asc2P-administered cells by coulometric ECD- or UV-HPLC methodology. Asc, Asc2P and DehAsc were separated as shown by retention times of 3.40–3.44, 6.48–6.54 and 39.5–41.7 min, respectively, under the chromatographic conditions employed (not shown). No time-dependent decrease in the chromatographic peak area during HPLC development was observed for initial amounts of authentic Asc or the derivatives dissolved in the cell extract. Extracts from Asc2P-administered cells contained abundant Asc but no Asc2P as an intact form, showing that Asc2P undergoing transmembrane uptake into the cell was promptly dephosphorylated and retained as an Asc form, but was scarcely oxidized into DehAsc. A dose-dependent saturated mode of intracellular Asc influx amounts was shown for administration of 50–200 μM Asc2P (Fig. 5). In contrast, only slight uptake was observed for Asc as low as



Color Plate I (See page 101, Figure 3) Fluorescence microphotographs demonstrating the cytoprotective effects of Asc2P against t-BuOOH-induced mortality of BAE-2 cells. Cells were exposed to t-BuOOH and/or Asc2P for 7 hr as described in Figure 2, and then fluorescence and normal microphotographs were taken. The scale indicates a length of 90 µm.

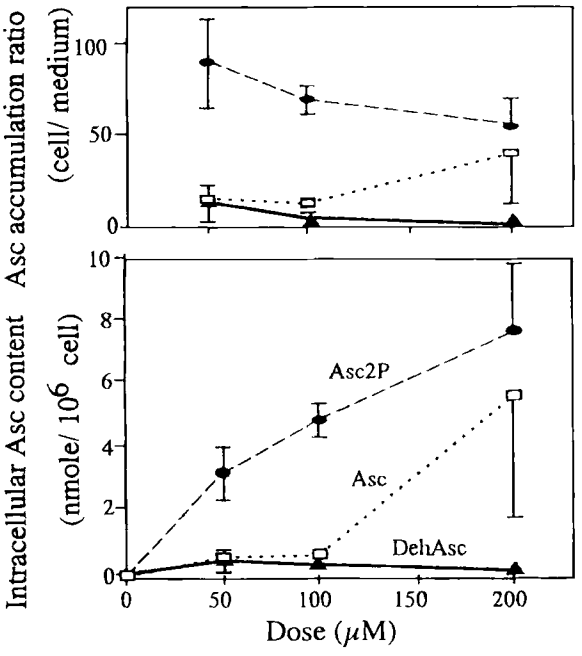


FIGURE 5 Dependence of Asc uptake into BAE-2 cells on doses of Asc or its derivative administered as described in Figure 2. Cells were administered with Asc/derivative of doses indicated in the presence of 50 μM dithiothreitol, affecting no significant difference except for DehAsc, which was slightly more effectively taken up in the presence than in the absence of the reductant. The intracellular Asc content was determined for cell extracts by coulometric ECD- and UV-HPLC methodology. The intracellular Asc accumulation ratio was defined as the molar ratio of intracellular Asc concentration versus extracellular Asc/derivative concentration. The intracellular Asc concentration was obtained as the quotient of intracellular Asc amount to intracellular water volume (Table I) determined by the [^{14}C]PEG/gas chromatographic method. The data were typical of four independent experiments. The bar represents the S.D. of wells in triplicate.

50–100 μM corresponding to an Asc concentration range in human normal serum, whereas a marked increase of influx was reproducibly observed for Asc as high as 100–200 μM. Administration of DehAsc as much as 200 μM resulted in no increase in intracellular Asc amounts, showing no appreciable uptake of DehAsc. Asc but scarcely DehAsc was detected for any extracts from cells administered with Asc, DehAsc or Asc2P, suggesting a highly reducing potential within the cell. The result was shown not to ensue from possible irreversible oxidative decomposition of DehAsc to 2,3-diketo-L-gulonic acid during extracting operation or HPLC development, because the major part of authentic DehAsc dissolved in the cell extract could be recovered in the presence or absence of dithiothreitol in the chromatogram under the conditions employed. Intracellular Asc accumulation amounts for Asc2P administration were 6.7- and 13-fold as large as those for its parent Asc administered at 50 and 100 μM, respectively.

Ratio of Intracellular Asc Versus the Extracellular Concentration

The intracellular water content of BAE-2 cells was 0.587 pL/cell as determined by gas chromatography using [^{14}C]PEG as a carrier that is not penetrable into cells (Table I).[15,16] Based on the value determined, Asc2P administered at 50 μM was estimated to accumulate as its dephosphorylated form within the cell to 4.6 mM, which was 90-fold higher than the extracellular concentration, showing a marked uphill influx of Asc2P into the cell against a concentration gradient (Fig. 5). In contrast, Asc or DehAsc administered was estimated to accumulate within the cell in the form of Asc to a level as low as 0.55 mM or 0.036 mM, corresponding to a 11- or 0.71-fold higher concentration, respectively, than the extracellular concentration of 50 uM. Thus accumulation at an intracellular Asc concentration as high as 90-fold was achieved by administration with Asc2P, but not with Asc or DehAsc.

DISCUSSION

Cytoprotective effects against peroxidants such as t-BuOOH and NDE have been examined for some endogenous biomolecules other than Asc.[18,19] The present study has demonstrated that cytoprotection against t-BuOOH was achieved by Asc2P but not Asc for the aortic endothelial cells through enrichment of intracellular Asc. UV-induced apoptosis of epidermal

TABLE I Determination of the intracellular water content of BAE-2 cells cell by [^{14}C]PEG/gas chromatography

Packed cells			Extracellular space		Intracellular water content		Intracellular water rate*
Number (cells) A	Weight (mg) B	Moisture (mm^3) C	Supernatant (mg) D	Moisture (mm^3) E	Total (mm^3) C-E	Per cell (pL/cell) (C-E)/A	(%) (C-E)/(B-D)
(2.90 ± 0.06) × 10^7	21.2 ± 0.2	17.97 ± 1.35	0.96 ± 0.02	0.93 ± 0.03	17.04 ± 0.24	0.587 ± 0.008	84.2 ± 1.2

*The intracellular water rate is defined as the proportion of the volume of intracellular water to the net wet weight of a cell. The values represent averages and S.D. typical of two independent experiments performed in quadruplicate.

keratinocytes is also prevented by Asc2P but not Asc although the mechanism has not been clarified.[17] Furthermore, Asc2P but not Asc inhibits the invasive motility of fibrosarcoma cells into reconstituted basement membrane[20] probably via scavenging of endogenous ROS necessary for tumor invasion.[21] Thus, some ROS-associated biological phenomena have been shown to be effectively controlled by Asc2P but not Asc, although the mechanism remains to be determined. The present study is the first describing a correlation between the cytoprotective effect of Asc2P and enrichment of intracellular free Asc.

Endogenous ROS scavengers other than Asc may be also involved in cytoprotection against peroxidation because artificial enrichment of intracellular Asc potently decreases the other scavengers so as to retain the total antioxidant ability of the cell to a defined level. However, the endogenous Asc content has been shown to be more closely associated with some ROS-related cellular processes than other endogenous scavengers.[22,23] For example, UV-irradiation of skin of hairless mice rapidly depletes endogenous Asc existing in the epidermis but not some other ROS scavengers.[22] In addition, piperidinoxyl radicals artificially generated in mouse skin homogenates are scavenged most efficiently by addition of Asc out of diverse antioxidants.[23]

A convex curve of Asc2P for doses versus influx amounts in contrast to a concave curve of Asc (Fig. 5) suggests superiority of Asc2P in terms of maintaining the adequate influx amount within a defined range. The controlled uptake of Asc2P is also observed for less time-dependent retention of serum Asc after oral administration of Asc2P to guinea pigs than of Asc.[24] The controlled uptake is rational for the organism because the requirement for Asc uptake is greater under conditions of Asc shortage than upon Asc abundance[25,26] also, because excess Asc is cytotoxic in the present of metal ions[7] or for sparsely growing cells.[12,13]

The mechanism whereby Asc2P undergoes efficient uptake remains to be determined with the use of [^{14}C]Asc2P, which can be prepared by enzymatic reaction of [^{14}C]Asc with pyrophosphate by phosphorylase extracted from *Pseudomonous azotocolligans*[27] or liver of rainbow trout,[28] suggesting a possibility that a trace of Asc2P may occur in vivo.

Acknowledgments

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