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Cytoprotection of Vascular Endotheliocytes by Phosphorylated Ascorbate Through Suppression of Oxidative Stress That Is Generated Immediately After Post-Anoxic Reoxygenation or With Alkylhydroperoxides

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Abstract Vascular endotheliocytes BAE-2 underwent the gradually proceeding cell death until 48 h after reoxygenation (Reox) following 3 h anoxia (Anox), but protected by pre-Anox administration with L-ascorbic acid (Asc)-2-*O*-phosphate (Asc2P), an autooxidation-resistant Asc derivative, but not by Asc itself. This cytoprotection with Asc2P was achieved in a glucose (Glc)-lacking buffer more advantageously than in a Glc-containing buffer where less efficiency had been demonstrated for Asc entry into BAE-2 cells than in a Glc-lacking buffer. Superoxide anion radicals were detected explosively in the extracellular space at 2–5 min after Reox following the Anox treatment of HUVE endotheliocytes, and were thereafter retained at levels as high as approximately one-half of the maximum level until 60 min after Reox, as shown by cytochrome c reduction assay. Superoxide anions at 3 and 60 min after Reox were suppressed by pre-Anox administration with Asc2P, but not with Asc or dehydro-Asc, and were not suppressed by post-Anox administration with Asc2P; the cytoprotection may need the intracellular accumulation of the ROS-scavenging effector Asc that is converted from Asc2P until 3 min after Reox. The ROS-generator tert-butylhydroperoxide (t-BuOOH) also induced both the diminished cell viability and nuclear DNA strand cleavages of BAE-2 endotheliocytes, which were also protected dose-dependently with Asc2P. The cytoprotection was attributed to reduction of intracellular ROS including hydroperoxide and hydrogen peroxide with Asc2P as shown by fluorometry with the redox indicator CDCFH-DA. Thus Anox/Reox-induced cell death can be prevented by Asc2P that suppresses ROS-generation immediately after Reox following Anox more efficiently in the intracellular sphere rather than in the extracellular space. J. Cell. Biochem. 93: 653–663, 2004.

Key words: L-ascorbic acid-2-*O*-phosphate; reactive oxygen species; anoxia and reoxygenation; tert-butyl hydroperoxide; cell death

Vascular endothelium is one of the major initial target tissues which reactive oxygen species (ROS) injures through oxidative reaction to cellular components. Post-ischemic reperfusion is regarded as an extensively occurring phenomenon in the mammalian living body and as a substantial cause for cell death and the subsequent senescence and vascular diseases [Liu et al., 2002]. As a suitable in vitro experimental model for post-ischemic reperfusion, a post-anoxic reoxygenation (Anox/Reox) model using the cultivated endothelial cells is known to be useful [Zweier et al., 1994; Cepinskas et al., 1999], and is attempted in the present study to be demonstrated for its advantages especially for screening some effective therapeutic agents for vascular diseases.

Out of some cytoprotective lead compounds against Anox/Reox-induced cell death, L-ascorbic acid (Asc) is known to defend the living

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tissues against damage by free radicals because of its antioxidant properties [Antonenkov and Sies, 1992; Makar et al., 1994]. Asc reacts readily with a variety of ROS including superoxide anion radical, singlet oxygen, hydroxyl radical, and water-soluble peroxyl radicals [Halliwell and Gutteridge, 1990]. Moreover, it has been reported that Asc and alpha-tocopherol synergistically play a role in the prevention of lipid peroxidation, as Asc readily reduces the tocopheroxyl radical at the membrane interface [Niki, 1991]. On the other hand, it is also known that Asc is such an unstable substance as is so easily oxidized and loses its antioxidant ability.

L-ascorbic acid-2-*O*-phosphate (Asc2P) is stably masked at the 2,3-enediol moiety, which retains the antioxidant ability of Asc more markedly than Asc itself does. Most portion (about 85%) of Asc2P is exempted from autooxidation, and retains the activity as vitamin C for 7 days after being dissolved in solution at 37°C, whereas Asc is easily oxidized, and loses its activity after only about 24 h [Hata and Senoo, 1989]. Recently, we demonstrated that Asc2P prevents ROS-related cellular injuries such as post-ischemic reperfusional injury in the liver [Eguchi et al., 2003a], hydrogen peroxide-induced cell death in cardiomyoblasts [Eguchi et al., 2003b], age-dependent telomeric DNA shortening in endothelial or epithelial cells [Furumoto et al., 1998], UV-B irradiational injury in the skin keratinocytes [Kanatate et al., 1995], lipid peroxide-induced injury in vascular endothelium [Fujiwara et al., 1997], and tumor invasion and metastasis [Nagao et al., 2000]. Although it is thought that these efficacies of Asc2P are attributed to its antioxidative activity, it has not been studied yet whether Asc2P can suppress ROS-induced injury through suppression of ROS generation in vascular endothelial cells, one of the major initial target tissues that are injured by ROS, which is generated during diverse pathological process such as post-ischemic reperfusion, inflammation, and atherosclerosis [McCord, 1987].

In the present study, we investigated the effects of Asc2P on ROS generation and ROS-induced cell injuries in the vascular endothelium by using two well established experimental models that have been extensively used for the in vitro studies of oxidative injury, Anox/Reox treatment [Saitoh et al., 2003b] and exposure to the chemical oxidant tert-butyl hydroperoxide (t-BuOOH), an analogue of short-chain lipid hydroperoxide intermediate that is formed during oxidative stress [Saitoh et al., 2003a].

MATERIALS AND METHODS

Cell Culture

Vascular endothelial cells BAE-2 obtained from bovine aorta were generously gifted by Dr. Kiyotaka Yamamoto of Tokyo Metropolitan Institute of Gerontology, and were used at population doubling levels of 29–59. Human umbilical vein endotheliocytes (HUVE) being mycoplasma-free were obtained from Kurabo Co., Osaka, Japan. BAE-2 cells were maintained in MEM supplemented with 10% dialized and heat-inactivated fetal bovine serum (FBS, Gibco-BRL, Rockville, MD). HUVE were maintained in DMEM supplemented with 20% dialized and heat-inactivated FBS. Cells were grown in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The subconfluent cells were treated for subculture with 0.25% trypsin–0.02% EDTA in phosphate-buffered saline (PBS), neutralized with an equivoluminal culture medium, and detached with a scraper. The cell number was determined with a Coulter electric particle counter ZM equipped with a channelyser model 256 (Beckman Coulter, Fullerton, CA).

Anoxia/Reoxygenation Experiment

Anoxia was created by incubating cells in a modular-incubator with a diameter of 35 cm (Billups-Rothenberg, Inc., Del Mar, CA) containing a deoxidizer Anaerocult A mini (Merck, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% N_2 and 5% CO_2. By replacing under the standard cell culture conditions (37°C, 95% air and 5% CO_2), the cells underwent reoxygenation.

Determination of Cell Viability

Cell viability was quantified by measuring mitochondrial dehydrogenase activity retained in the cultured cells by photometric assay using the formazan-forming dye WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium, sodium salt; Wako, Osaka, Japan]. Briefly, cells were cultured in 96-well flat bottomed plates with phenol red-free medium. At selected points, 30 μl of solution containing

5 mM WST-1 and 200 μM 1-methoxy PMS was added to a well of the microplate, and the cells were incubated for 2 h at 37°C. Then, absorbance at 450 nm relative to the reference wavelength of 650 nm was measured with a Bio-Rad microplate absorbance reader model 3550 (Bio-Rad, Hercules, CA). The cell viability is expressed as a percentage of that of control cells ($100 \times A_{450,\ treated}/A_{450,\ control}$). Furthermore, cell viability was also evaluated by Trypan Blue Exclusion Assay. At selected points, cells were trypsinized, resuspended in 0.5 ml of Hanks' balanced salt solution (HBSS), and stained by adding 0.5 ml of 0.4% trypan blue for 5 min. All the stained and unstained cells were counted in 4 squares of a hemocytometer and the percentage of cell death calculated.

Measurement of Released Superoxide Anion Level

Levels of superoxide anion radials released from HUVE were quantified by cytochrome c reduction method. Two hundred microliters of culture media was subjected to 96-well microplate and bovine heart cytochrome c (Wako) was added at a final concentration of 90 μM, with or without Cu/Zn superoxide dismutase (SOD; Wako) at a final concentration of 300 U/ml. The absorbance of cytochrome c was measured at 550 nm with a microplate absorbance reader. The difference between paired plates with and without SOD was taken as an SOD-inhibitable cytochrome c reduction value. The actual amount of O_2^- were assumed with a molar extinction coefficient of $18.5 \times 10^3\ M^{-1}cm^{-1}$.

In Situ Detection of Cellular Oxidative Stress

Cells were grown in the presence or absence of Asc2P for 18 h, then treated with or without the membrane-permeant peroxidizing agent t-BuOOH (Wako) for 3 h, rinsed twice with PBS and replaced by phenol red-free medium with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (CDCFH-DA, Molecular Probes, Eugene, OR). After incubation for 20, 40, or 90 min, the supernatant was transferred into a new microplate to quantify the extracellular oxidative stress. Fresh medium without phenol red was added to the platewell for measurement of the intracellular oxidative stress. The fluorescence intensity was measured with a fluorescence plate reader CytoFluor 2350 (Millipore, Bedford, MA).

TUNEL Assay

DNA fragmentation of individual cells was detected in situ by TUNEL (Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling) with the In situ Apoptosis Detection Kit (Takara, Kyoto, Japan). Cells grown on coverslips (2.4×10^4 cells in a 35 mm dish) were washed with PBS and fixed in paraformaldehyde solution (4% in PBS) for 20 min at room temperature. The cells were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase. Then the cells were permeabilized in a solution containing 0.1% Triton X-100 for 5 min on ice, followed by incubation in freshly prepared TUNEL reaction mixture for 90 min at 37°C in the dark. The coverslips were washed with PBS and mounted on slides with antifading solution. TUNEL staining was analyzed with a confocal laser fluorescence microscope (Bio-Rad).

Statistical Analysis

Data were presented as mean ± SD, and statistical comparisons were performed using a unpaired Student's *t*-test or Dunnet's multiple comparison test (StatView IV; Abacus Concepts, Inc., Berkley, CA). Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Induction of Cell Death by Anox/Reox Treatment in Aortic Endothelial Cells

Bovine aortic endothelial cells BAE-2 were subjected to anoxic treatment (95% N_2, 5% CO_2) for 3 h and then were placed under normoxic conditions. This Anox/Reox obviously induced diminishments of both cell number (Fig. 1a) and trypan blue-excluding cell viability in contrast to no diminishment for the control (undergoing no Anox/Reox treatment) (Fig. 1b). Cell death was increased in a time-dependent manner. At 48 h after outset of Reox, BAE-2 cells suffered from extensive cell death.

Effect of Asc2P on Anox/Reox Induced Cell Death

The Anox/Reox-induced cell death has been shown to result from cell injuries appreciably attributed to intracellular ROS, suggesting the preventive effects of antioxidative agents on cell death. We tried to introduce Asc resulted in no

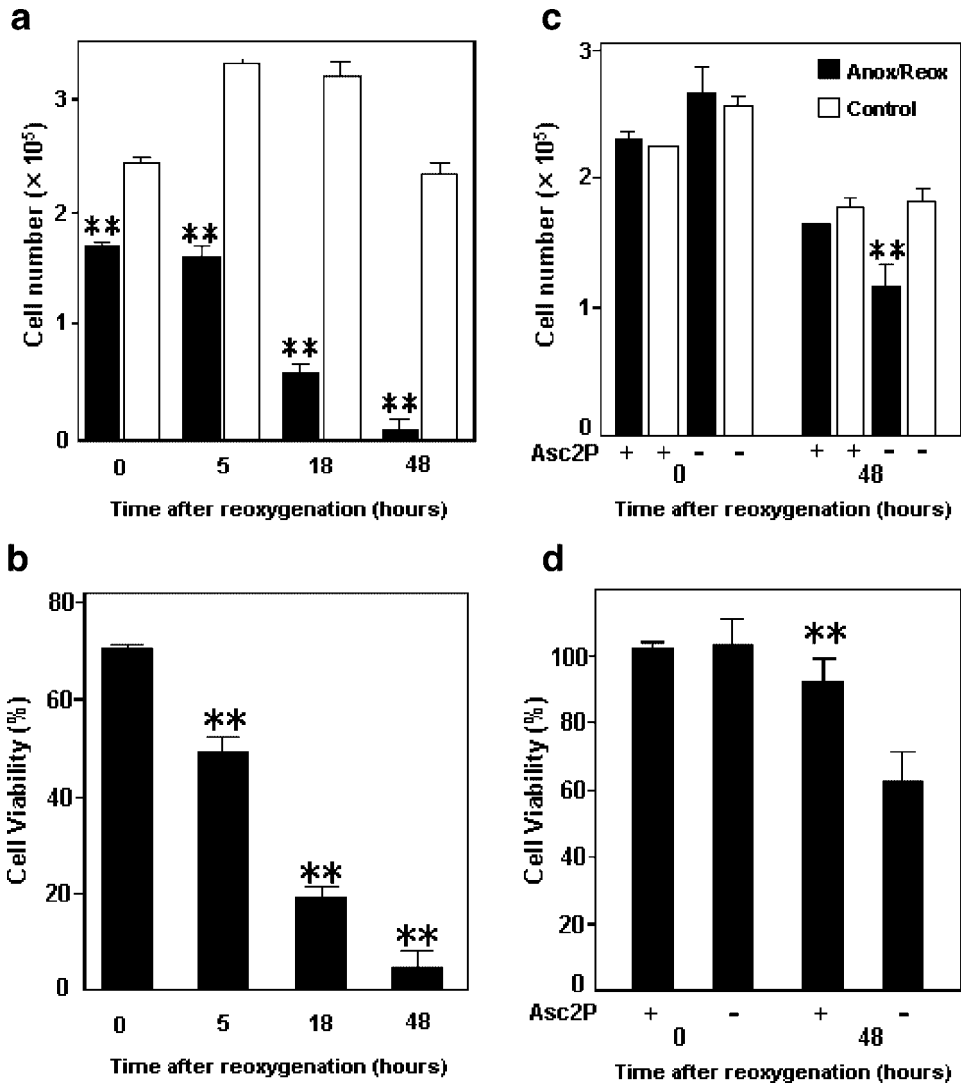

Fig. 1. Anox/Reox-induced cell death (**a**, **b**), and cytoprotection against Anox/Reox-induced cell death by Asc2P treatment (**c**, **d**) in bovine aortic endothelial cells BAE2. BAE2 cells were precultured in culture medium (a, b) or with vehicle or Asc2P (200 μM) (c, d) for 18 h. Culture medium was replaced by HBSS (a, b) or HBSS containing vehicle or Asc2P (200 μM) (c, d) immediately before anoxia treatment (95% N_2, 5% CO_2). Cells were placed under anoxic conditions for 3 h and then underwent reoxygenation by placing under normoxic conditions. After reoxygenation started, cells were collected at indicated times by trypsinization and centrifugation, and counted with an electric particle counter. $^{**}P < 0.01$ versus control (unpaired Student's *t*-test) (a), $^{**}P < 0.01$ versus Asc2P addition (unpaired Student's *t*-test) (c). Cell viability was estimated by trypan blue dye exclusion method, and expressed as the rate versus a value under normoxic conditions $^{**}P < 0.01$ versus time 0 (Dunnet's multiple comparison test) (b), $^{**}P < 0.01$ versus Asc2P addition (unpaired Student's *t*-test) (d). The data shown were typical of three independent experiments with dishes in triplicate.

success in marked cytoprotection, and secondly to introduce the autooxidation-resistant type of Asc, Asc2P. When Asc2P at 200 μM was previously introduced before Anox/Reox treatment, the cell viability was restored up to almost the same level versus that without Anox/Reox treatment (Fig. 1c,d). This might be due to scavenging of intracellular ROS by introduced Asc2P.

To clarify the preventive effect of introduced Asc2P on Anox/Reox-induced cell death, we tried to introduce Asc2P at some concentrations in the presence (HBSS) or absence (PBS) of coexistent glucose (Fig. 2a–d). This is because of uptake of extracellular Asc across the cell membrane is dose-dependently inhibited by coexistent glucose [Saitoh et al., 1997].

Under Anox/Reox conditions in the presence of coexistent glucose (Fig. 2a,b), cell viability at 42 h after outset of Reox was lowered to approximately 40% versus the untreated control,

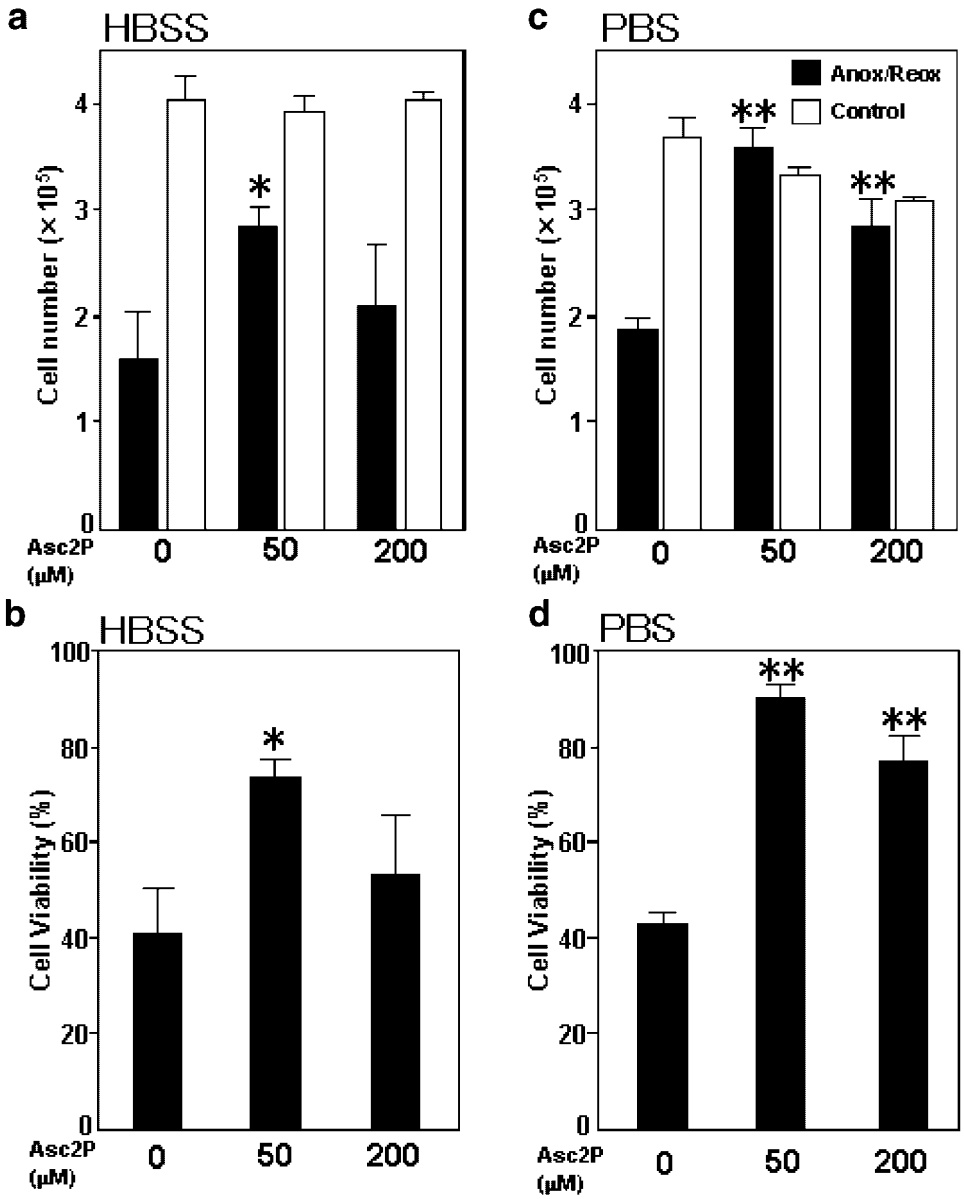

Fig. 2. Dose-dependency of cytoprotection against Anox/Reox-induced cell death by Asc2P treatment. BAE2 cells were pre-treated with vehicle or Asc2P (50 and 200 μM) for 18 h. Culture medium was replaced by HBSS (**a** and **b**) or PBS (**c** and **d**) containing vehicle or Asc2P immediately before anoxia treatment. Cells were placed under anoxic conditions for 3 h and then underwent reoxygenation by placing under normoxic conditions. At 42 h (a and b) or 15 h (c and d) after reoxygenation started, cells were collected and counted similarly as done in Figure 1a and c. Cell viability was estimated and expressed as in Figure 1b and d. The data shown were typical of three independent experiments with dishes in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ versus no Asc2P addition (unpaired Student's *t*-test).

whereas previous addition of Asc2P before Anox/Reox treatment made cell viability to be recovered to 75%. In contrast, glucose-lacking conditions (Fig. 2c,d) resulted in cell death occurring as early as 15 h after Reox, whereas cell viability was recovered up to the level as high as approximately 90% upon previous addition of Asc2P at 50 μM. These results (Fig. 2a–d), by which coexistent glucose moderately depresses the cytoprotective effect of Asc2P against Anox/Reox-induced injuries, suggest that it is important for the efficacy of Asc2P to be taken in into a cell as Asc. In addition, the cytoprotection by antioxidant agents such as the Asc derivative suggests that Anox/Reox-induced cell death was substantially triggered by cellular ROS.

Scavenging of Superoxide Anion Radicals That Are Released From Vascular Endothelial Cells by Anox/Reox Treatment

To analyze the mechanism underlying the cytoprotection by Asc2P against Anox/Reox-induced cell death, generation and diminution of ROS, especially superoxide anion radicals, were measured. This was conducted with the use of endothelial cells HUVE derived from human umbilical vein, because of the final aim for clinical application, instead of endotheliocytes from bovine species that are advantageous to extensive examination for cell viability. We tried to confirm the levels of superoxide anions released into the extracellular space from Anox/Reox-treated cells by cytochrome c reduction method. Figure 3a clearly indicated that transient explosive generation of superoxide anion radicals was detected in the extracellular space at 2–5 min after Reox following Anox of HUVE cells, and the level of released superoxide anions was sustained to be as abundant as approximately one-half of the maximum level until a period of 60 min after Reox. The results suggest that Anox/Reox-induced cell death was attributed appreciably to the intracellular ROS that was explosively generated immediately (0–2 min) after Reox before detection (shorter than 2 min) of extracellular ROS.

We next examined the effect of Asc2P on the Anox/Reox-induced release of ROS from HUVE at both the time points of 3 min (the acute period) and 60 min (the steady period) after Reox started (Fig. 3b). Allopurinol, an inhibitor of xanthine oxidase, effectively inhibited the production of superoxide anions. Cu/Zn-SOD, a scavenger of superoxide anions, completely inhibited acute release and moderately inhibited constant release. The results indicate that the detection of superoxide anions by cytochrome c reduction method employed here was substantially attributed to both generation of superoxide anions through intracellular events including xanthine oxidase reaction and subsequent release into the extracellular space for which macromolecules such as SOD were accessible.

Previous administration of Asc or dehydroascorbic acid (DehAsc) gave a promotive or no significant effect on production of superoxide anions induced by Anox/Reox treatment. Asc or DehAsc that was previously administered was suggested to be oxidatively degraded and thereafter to act as an pro-oxidant. In contrast, previous administration with Asc2P reduced the level of superoxide anions at both time points. This reducing effect of Asc2P at 40 μM was more or not less marked than the effect of allopurinol at 3 or 60 min after outset of Reox, respectively. Post-anoxic administration with Asc2P at both 40 and 120 μM, however, achieved no or only a moderate diminution of superoxide anions at 3 or 60 min after outset of Reox, respectively. This suggests both the insufficient introduction of the antioxidant into cells during a time as short as 3 min after Asc2P administration and the requirement for approximately 1 h of preincubation in order to exert an ROS-scavenging activity assumedly through enzymatic conversion to an Asc free acid form from Asc2P. The results indicate that Anox/Reox treatment induced xanthine oxidase-mediated ROS production, and Asc2P can scavenge ROS responsible for Anox/Reox-induced cell death.

Effect of Asc2P on Oxidative Stress Induced by the Membrane-Permeable Peroxidant t-BuOOH

Anti-oxidative effect of Asc2P was further examined by imposition of oxidative stress with t-BuOOH, a membrane-permeable peroxidant, instead of Anox/Reox treatment. Treatment with t-BuOOH clearly induced reduction of cell viability of endotheliocytes BAE-2. At concentrations over 100 μM, cell viability was greatly reduced to less than 10% versus that of the untreated control, and, at 50 μM, cell viability was lowered to about 35% (Fig. 4a). Cells that were previously administered with Asc2P at 50–150 μM appreciably restored the t-BuOOH-induced diminution of cell viability. At a t-BuOOH concentration of 100 μM, cell viability was recovered to levels as high as 70–80% by Asc2P at 100 or 150 μM in contrast to 10% for treatment without Asc2P. Only modest recovery was seen, however, for treatment with t-BuOOH as high as 125 μM. This might be due to such extensive cell injuries as cannot be prevented assumedly owing to a definite yielding rate of Asc to which Asc2P is esterolytically converted.

We next tried to confirm the involvement of ROS scavenging in the mechanism of cytoprotection by Asc2P. Intracellular ROS including H_2O_2 and other peroxides was measured by incubation with CDCFH-DA, a peroxide-susceptible fluorescence dye, which becomes brightly fluorescent after oxidative conversion to CDCF

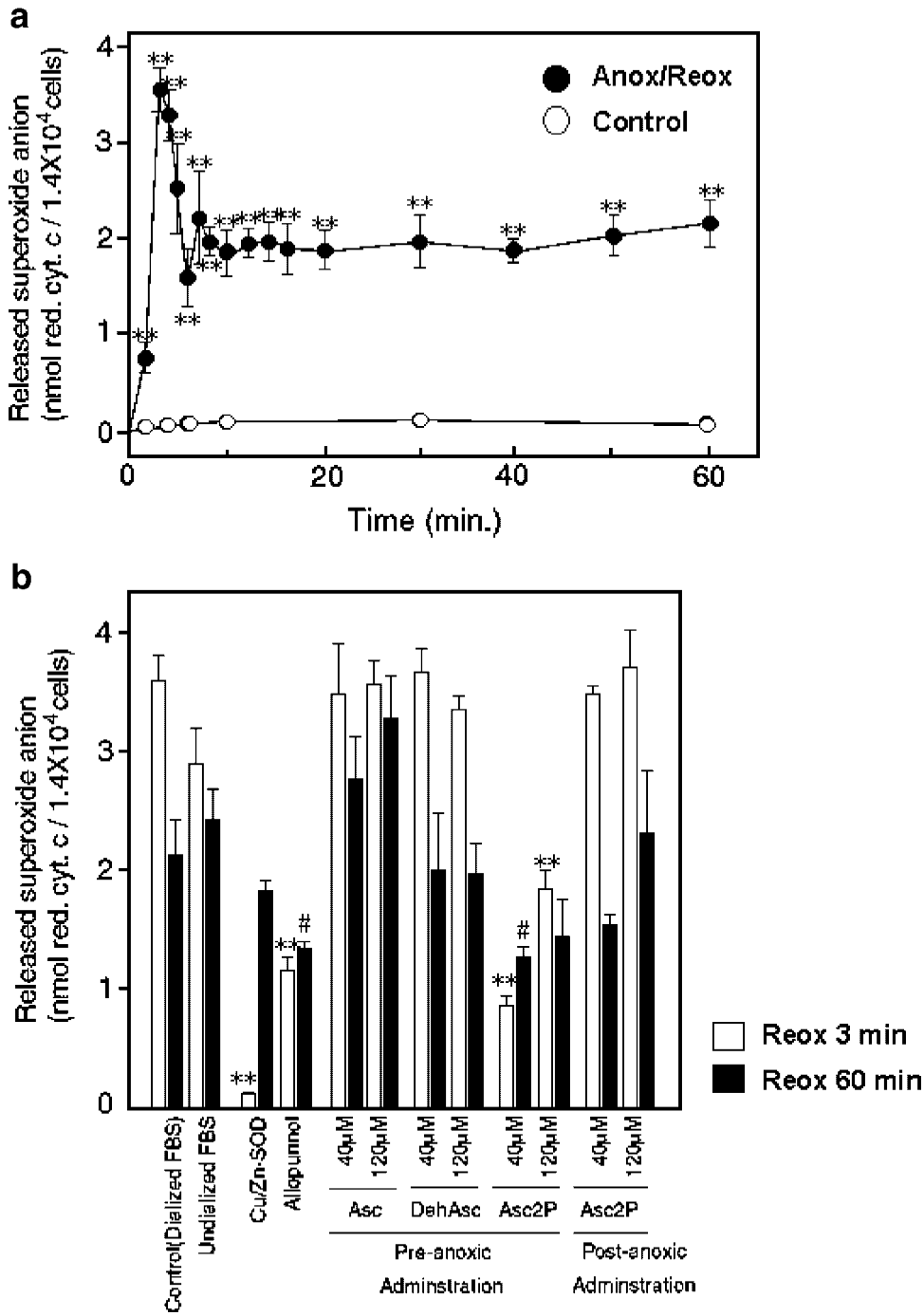

Fig. 3. **a**: Time course of the level of superoxide anion radicals increasingly released from human umbilical vein endotheliocytes HUVE by Anox/Reox treatment. HUVE were anoxically treated for 2 h and then underwent reoxygenation by placing under normoxic conditions. Some portions of culture medium were subjected to measurements of the released superoxide anion level by cytochrome c reduction method at indicated times after outset of reoxygenation. The data shown were typical of four independent experiments with dishes in triplicate. $^{**}P < 0.01$ versus control (unpaired Student's *t*-test). **b**: The repressive effects of treatment with derivatives of ascorbic acid on the increased level of superoxide anion radicals that were induced by Anox/Reox treatment HUVE were treated with vehicle or ascorbic acid derivatives (40 and 120 μM), which were previously administered (18 h before anoxia treatment) or later administered (immediately after termination of anoxia treatment). Cells were anoxically treated for 2 h and then underwent reoxygenation by placing under normoxic conditions. At 3 and 60 min after reoxygenation started, culture medium was subjected to estimation of the level of released superoxide anion radicals by cytochrome c reduction method. The data shown were typical of three independent experiments with dishes in triplicate. $^{**}P < 0.01$ versus Reox 3 min, $^{\#}P < 0.05$ versus Reox 60 min (unpaired Student's *t*-test).

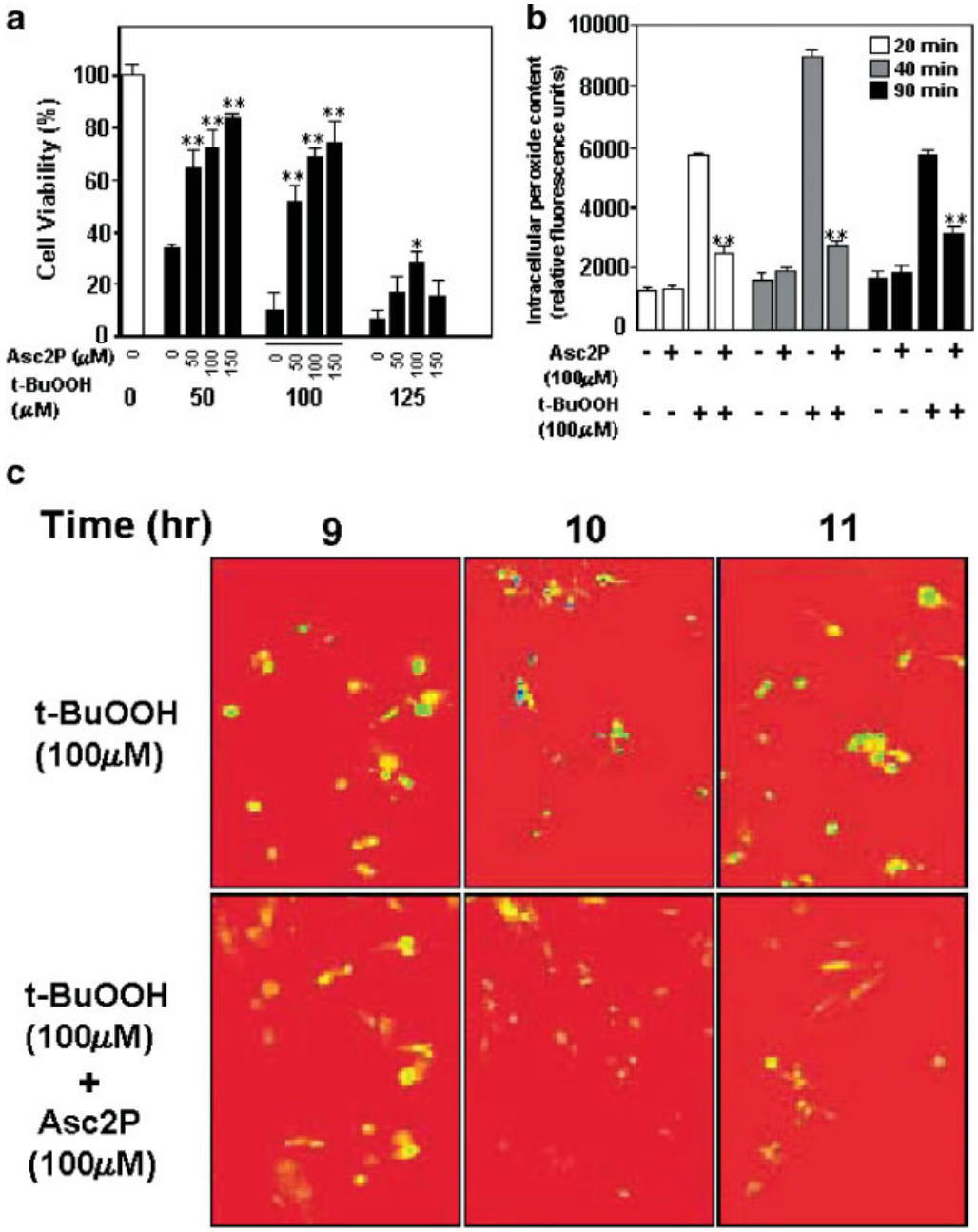

Fig. 4. **a**: Dose-dependent prevention by Asc2P from t-BuOOH-induced cell death. BAE2 were pre-treated with vehicle or Asc2P (50, 100, and 150 μM) for 18 h. Then, vehicle or t-BuOOH (50, 100, and 125 μM) was added to culture medium and incubated for further 24 h. Cell viability was estimated by WST-1 method using a formazan-forming redox indicator, and expressed as the rate versus a value for vehicle treatment. The data shown were typical of three independent experiments with wells in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ versus no Asc2P addition (unpaired Student's *t*-test). **b**: Reduction by Asc2P of a t-BuOOH induced increase of the intracellular peroxide level BAE2 were pre-treated with vehicle or Asc2P (100 μM) for 18 h. Then, vehicle or t-BuOOH (100 μM) was added to culture medium and incubated for further 3 h. The intracellular level of peroxides including H_2O_2 was estimated by fluorometric assay using the peroxide-susceptible fluorescence dye CDCFH-DA. Periods of incubation with CDCFH-DA (20, 40, and 90 min) were indicated. The data shown were typical of three independent experiments with wells in triplicate. $^{**}P < 0.01$ versus no Asc2P addition (unpaired Student's *t*-test). **c**: Prevention of t-BuOOH induced cleavages of cellular DNA strands by treatment with Asc2P BAE2 were grown on wells of a slide chamber and pre-treated with vehicle or Asc2P (100 μM) for 18 h. Then, t-BuOOH (100 μM) was added to culture medium in the presence of Asc2P and further incubated. At 9 h after t-BuOOH addition, cells were fixed at each interval of 1 h and stained by TUNEL method. Fluorescent signals were visualized by laser confocal fluorescence microscopy. The fluorographs shown were typical of three independent experiments and selected as the average field out of eight microscopic fields per a well.

by intracellular ROS such as peroxides (Fig. 4b). Cells that were treated with t-BuOOH alone greatly increased the level of intracellular ROS, whereas Asc2P-added cells diminished the intracellular ROS to nearly the control level. These data clearly indicate that recovery of cell viability by Asc2P ensued from scavenging of ROS that was secondarily produced by t-BuOOH.

Preventive Effects of Asc2P on t-BuOOH Induced DNA Cleavage

We tried to characterize by TUNEL assay whether t-BuOOH induced cell death is apoptosis or not. Fluorescence intensity for TUNEL stain was expressed as diverse pseudo-colors. Dark blue or green in the nuclei of BAE-2 cells that were treated with t-BuOOH for 9–11 h, respectively, represents the markedly or appreciably extensive DNA cleavages (Fig. 4c, the upper panels), whereas cells that were previously administered with Asc2P and then treated with t-BuOOH showed the diminished DNA cleavages as indicated by yellowish pseudocolor (Fig. 4c, the lower panels). These data indicate that t-BuOOH induced cell death might be apoptosis-like and suggests that it ensued from DNA cleavages mediated by ROS which could be appreciably scavenged by Asc2P.

DISCUSSION

Anoxic treatment (Anox) of vascular endothelial cells, even for the defined period, was found to induce thereafter the persistent progress of cell death during a subsequent reoxygenation (Reox) period as long as 48 h (Fig. 1a,b) probably through a long-term cascade chain reactions within a cell. This Anox/Reox-induced cell death was shown to be attributed not only to a transient explosive generation of ROS such as superoxide anion at an acute period as short as 2–3 min after Reox, but also to a persistent ROS generation until at least 60 min after Reox in contrast to scanty of ROS below detection sensitivity limit for the control normoxic endotheliocytes (Fig. 3a). The scavenging of Anox/Reox-induced ROS at 3 and 60 min after Reox was accomplished by pre-anoxic administration with the phosphorylated derivative (Asc2P) of Asc but not with Asc or DehAsc (Fig. 3b), suggesting that a molecular property with resistance against oxidative decomposition is endowed with Asc2P but not with Asc itself and DehAsc, both of which are too labile to undergo the uptake into an intracellular space where Anox/Reox-induced ROS is generated and injures intracellular biomolecules. An ROS-scavenging ability of Asc2P was shown to be exhibited upon pre-anoxic administration but not upon post-anoxic administration (Fig. 3b), suggesting that conversion of the antioxidant precursor Asc2P to the active principle Asc demands the defined period necessary for dephosphorylation reaction with endogenous phosphatase as previously demonstrated [Fujiwara et al., 1997]. The Anox/Reox-induced ROS enables peroxidation of cellular lipids that is mediated via short chain lipid intermediates such as t-BuOOH, which was shown to also diminish the viability of endotheliocytes dose-dependently (Fig. 4a) via generation of intracellular ROS (Fig. 4b) and to be counteracted for either cell mortality or ROS generation by Asc2P. Thus, Anox/Reox-induced cell death in endotheliocytes can be prevented by pre-anoxic administration with Asc2P via efficient scavenging of ROS generated immediately and persistently after Reox, but not with Asc or DehAsc or by post-anoxic administration with Asc2P, each of which cannot achieve the persistent or sufficient supply of Asc that undergoes the intracellular uptake.

Numerous studies demonstrated that the generation of ROS is a key causative event in oxidative injury and cell death, and that ROS is implicated in pathogenesis of many diseases [Halliwell et al., 1992; Halliwell and Gutteridge, 1994]. It has been indicated that Asc, one of the major water soluble antioxidative substances [Halliwell and Gutteridge, 1990], promptly scavenges ROS at an initial stage of ROS generation, because plasma lipoproteins exposed to aqueous peroxy radicals undergo no hydroperoxidation until depletion of endogenous Asc, which is depleted more rapidly and earlier than other plasma ROS-scavengers such as SH groups, alpha-tocopherol, bilirubin, and urate, suggesting that Asc efficiently protects biomolecules including lipids from oxidative damage [Frei et al., 1988]. Actually, many epidemiological and clinical studies have suggested that Asc is effective for various ROS-related pathological situations, such as cardiovascular diseases [Gey et al., 1987; Enstrom et al., 1992; Kelly, 1998; Frei, 1999], cataract [Varma and Richards, 1988; Bunce et al., 1990], and cancer [Bissell et al., 1980; Leung et al., 1993;

Kageyama et al., 1995; Wells et al., 1995; Takenaga et al., 1999].

In this study, we demonstrated that Asc2P, one of the derivatives of Asc, was reinforced for anti-oxidization potentials rather than Asc, and could exert excellent cytoprotective effects against ROS-mediated cellular injury models such as Anox/Reox [Kumar et al., 1996; Wu et al., 1998] and t-BuOOH treatment [Chance et al., 1979; Adams et al., 1996; Fujiwara et al., 1997; Saitoh et al., 2003a] through suppression of ROS generation in vascular endothelial cells. Furthermore, our results showed that Asc2P suppressed Anox/Reox-induced generation of ROS such as superoxide anion compared to Asc or DehAsc. The result is consistent with our previous reports that Asc2P is superior to Asc in terms of cytoprotective ability against lipid peroxide-induced injury in the vascular endothelium [Fujiwara et al., 1997], and UV-B irradiational injury in the skin [Kanatate et al., 1995], and tumor invasion and metastasis [Nagao et al., 2000]. We thought that this superior cytoprotective effect of Asc2P is ascribed to not only its stability, but also the enhancement of an intracellular content of Asc scavenging the endogenous ROS necessary for oxidative injuries and cell death, because we previously also demonstrated that Asc2P was accumulated as a dephoshorylated Asc form into vascular endothelial BAE-2 cells to an intracellular concentration rate of 70- to 90-fold relative to the extracellular Asc2P concentration, whereas Asc was 8- to 13-fold [Fujiwara et al., 1997]. Taken together, Asc2P, which potentiates antioxidative activity of Asc via intracellular accumulation, may exert excellent therapeutic effectiveness in a variety of ROS-related disorders.

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