Ascorbic Acid Enhances Nitric Oxide Production in Trabecular Meshwork Cells

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Purpose: This study investigated the role of ascorbic acid on the production of nitric oxide (NO) in the trabecular meshwork (TM) cells.

Methods: After primarily cultured human TM cells were exposed to 1, 10, and 100 μM of L-ascorbic acid (LAA), with or without co-administration of 1 mM sodium nitroprusside or 100 μM hydrogen peroxide for 48 hr, cellular survival and NO production were measured with MTT and Griess assay, respectively.

Results: LAA significantly potentiated NO production in a dose-dependent manner ($p<0.05$) without affecting cell viability. LAA increased cell viability after hydrogen peroxide-induced oxidative stress in a dose-dependent manner. LAA enhanced NO production in TM cells and showed a cytoprotective effect against hydrogen peroxide-induced oxidative stress.

Conclusions: LAA might be involved in the regulation of trabecular outflow by enhancing NO production in TM cells. *Korean Journal of Ophthalmology 19(3):227-232, 2005*

Key Words: Ascorbic acid, Hydrogen peroxide, Nitric oxide, Trabecular meshwork cell

Nitric oxide (NO) is a short-lived free radical, generated from L-arginine by NO synthases in an enzymatic reaction. It is diffusible across the biological membrane and has such diverse roles as an endothelium derived relaxing factor, a neuromodulator, and an immunological mediator in the cardiovascular, nervous, and immune systems.[1,2] In the eye, NO is a physiological regulator as well as a pathological mediator not only in the vascular system but also in other tissues including the retina and the ocular surface.[3] It has been proposed that the pathogenesis of primary open-angle glaucoma may be related to the alteration in the trabecular meshwork (TM), the ocular tissue believed to be responsible for the majority of aqueous humor outflow resistance. TM cells perform a variety of activities in maintaining the normal function of the aqueous flow pathway.

The activity of NO synthase has been shown in the TM of bovine, porcine and human eyes.[4-6] It is also known that NO is involved in the regulation of trabecular outflow and external administration of NO donor results in decreased intraocular pressure (IOP).[7,8] Alteration of NO synthase activity is reported in the glaucomatous eyes[9] and basal NO production is enhanced by hydraulic pressure in cultured human trabecular cells.[10]

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Ascorbic acid, the concentration of which is 15 times higher in the aqueous humor than the plasma,[11] is the major contributor to the antioxidant activity of aqueous humor.[12] Besides its well-known antioxidant property, ascorbic acid prevents endothelial dysfunction by potentiating NO synthesis in endothelial cells.[13,14] Endothelial dysfunction is characterized by an inability of endothelial cells to produce an adequate amount of bioactive NO.[15]

Taken together, there is a possibility that ascorbic acid, which exists in relatively high concentration in aqueous humor, can affect on the formation of NO in TM cells. The present study was designed to investigate whether ascorbic acid affects NO production in human TM cells. Additionally, the ability of ascorbic acid to prevent sodium nitroprusside-induced or hydrogen peroxide-induced damages to the viability of TM cells is also evaluated.

Materials and Methods

1. DPPH radical scavenging activity

In order to measure the antioxidant activity of ascorbic acid, the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was carried out according to the previously described procedure.[16] L-ascorbic acid (LAA: Sigma, St. Louis, MO, USA) at various concentrations was added to a 1.5×10^{-4} M solution of DPPH (Sigma, St. Louis, MO, USA) in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm,

and the radical scavenging activity was obtained from the following equation:

Radical scavenging activity (%) =

{(ODcontrol − ODsample) / ODcontrol}×100

The antioxidant activity of LAA was expressed as IC_{50}. The IC_{50} value was defined as the concentration of LAA that inhibits the formation of DPPH radical by 50%.

2. Cell culture

TM cell cultures were established from enucleated human eyes obtained from an eye bank and were transported on ice within 2 hr after exsanguinations as previously described.[17,18] Briefly, TM tissues were excised by dissecting a continuous strand of tissue between the line of Schwalbe and the scleral spur, using a curette to separate a continuous strand of TM tissue away from the scleral spur, ciliary muscle and Schwalbe's line. The excised TM tissues were placed in the sterile laminin-coated culture dish with DMEM/F-12 medium containing 15% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml gentamicin and 2.5 mg/ml fungizone, and were left undisturbed for 3 to 5 days in a 37℃ incubator with a 5% CO_2 atmosphere. After identifying the initial cell growth, the explants were removed and the cultures were maintained with a medium containing 10% FBS. The experimental results were based on passages three through five.

3. Experimental treatment

With approaching confluency, the cultures were trypsinized and inoculated into 24-well culture plates (1×10^6 cells/well). After allowing 24 hr for attachment, 1, 10, and 100 μM of freshly prepared LAA were added to the serum-free media with or without NOS inhibitor, N^{ω}-Nitro-L-arginine methyl ester (L-NAME: Sigma, St. Louis, MO, USA). In addition, 1 mM sodium nitroprusside or 100 μM hydrogen peroxide (diluted with phosphate buffered saline [PBS] at pH, 7.4) was administrated to the medium for 1 hr, with or without co-exposure of LAA. The cultures were replaced to the incubator and incubated for 48 hr.

4. Cell viability

Cell viability was determined by a rapid colorimetric assay using 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT: Sigma, St Louis, MO, USA). The MTT assay is based on the ability of the tetrazolium salt MTT to detect living but not dead cells. The signal generated, optical density (OD) is directly proportional to the number of cells.[19,20] After adding 100 μl of an MTT stock solution (5 mg MTT/ml PBS) to each well, the media was completely removed from the well after a 4-hr incubation at 37℃. Then 0.5 ml of dimethyl sulfoxide (DMSO: Sigma, St Louis, MO, USA) was added to each well and 100 μl of the solution from each well was transferred to a 96-well plate and read at 570 nm on a multi-well scanning spectrophotometer. It was noted that a minimum 10-min exposure to DMSO was required to dissolve the MTT formazan crystals after which the absorbance remained constant for 20 min. Thus, in all experiments spectrophotometric readings were taken 15 min after the addition of DMSO.

5. Measurement of NO production

Nitrite's concentration in the media was measured by Griess reaction.[21] Briefly, media samples were collected from each well following appropriate treatment and reacted with modified Griess reagent (Sigma, St Louis, MO, USA) by mixing equal volumes with each other at room temperature for 15 min. OD was then measured and read on a multi-well, scanning spectrophotometer at 540 nm. The nitrite concentration was then determined from a comparison of absorbance with that of a standard solution of sodium nitrite in medium. The background absorbance measured using the medium alone, was subtracted from all values.

6. Statistical analysis

Data are expressed as mean±standard error of the mean (mean±SEM) corresponding to the number of wells analyzed. Experimental differences between control culture results and a single treatment group were evaluated using Student's t-test and *P* values less than 0.05 were considered significant.

Results

1. DPPH radical scavenging activity of LAA

The antioxidant activity of LAA was evaluated by DPPH free radical scavenging activity. LAA showed relatively high DPPH free radical scavenging activity in a dose dependent manner, with an average IC_{50} value of 4.106 μM. In addition, after the standard sodium nitrite at various concentrations was mixed with LAA, nitrite concentration measured with Griess assay was dose dependently decreased (data not shown).

2. Cell culture

The cultured human TM cells were similar in appearance to those human TM cells generated by other investigators and identification of TM cells was done by their characteristic morphology and growth pattern.[17,18] Confluent cultures of TM cells grew as a monolayer of closely packed cells with branching cell bodies making multiple contacts between cells. The cells were flattened with rounded to moderately elongated bodies with each cell containing a centrally placed nucleus. Often the migrating TM cells showed ruffled borders as they detached from the main cell mass. Occasionally migrating cells formed satellite colonies of cells with

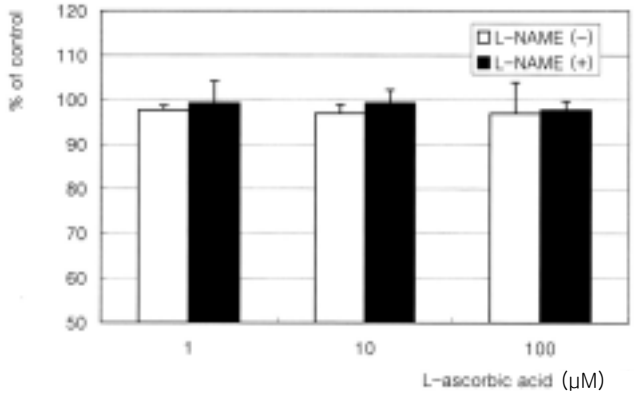

Fig. 1. Effect of L-ascorbic acid on the viability of cultured trabecular meshwork cells. L-ascorbic acid does not affect significantly on the viability of trabecular meshwork cells up to 100 μM.

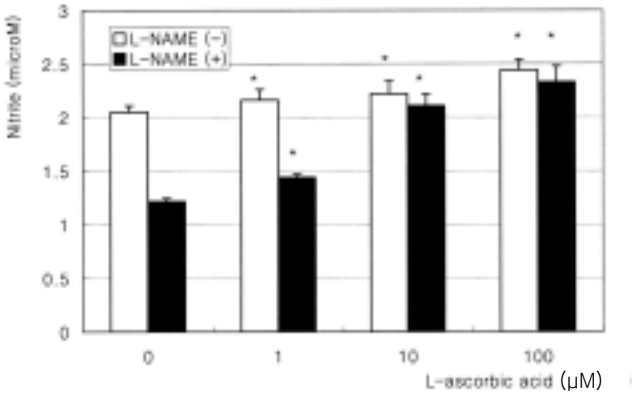

Fig. 3. Effect of L-ascorbic acid on the production of nitrite in cultured trabecular meshwork cells. L-ascorbic acid potentiate nitrate production significantly in a dose-dependent manner and L-NAME inhibited nitrite production significantly ($^{*}p<0.05$).

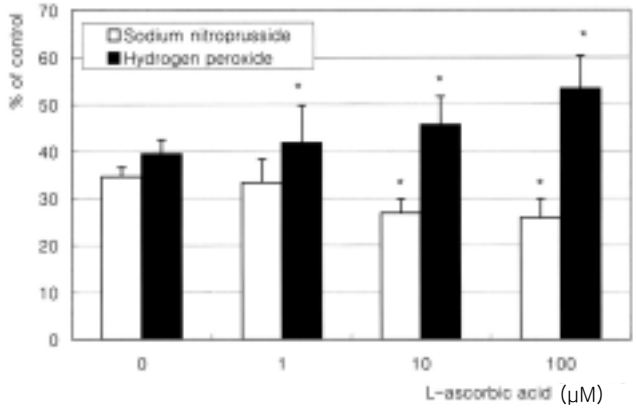

Fig. 2. Effect of 3 mM sodium nitroprusside and 1 mM hydrogen peroxide on the survival of cultured trabecular meshwork cells ($^{*}p<0.05$).

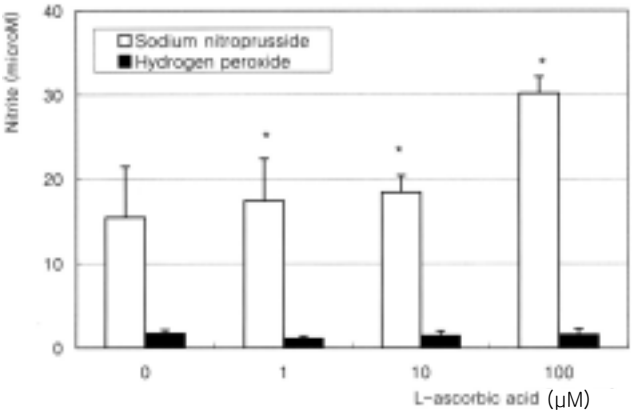

Fig. 4. Effect of L-ascorbic acid on the production of nitric oxide exposed to 3 mM sodium nitroprusside and 1 mM hydrogen peroxide in cultured trabecular meshwork cells ($^{*}p<0.05$).

identical morphology to the main cell mass.[17,18] This cellular morphology was maintained for at least 5 passages.

3. Effect on cell viability

LAA up to 100 μM did not significantly affect the survival of cultured TM cells (Fig. 1). Thus the effect on NO production of LAA at these concentrations was not influenced by cell numbers. Sodium nitroprusside at 1 mM markedly decreased cellular survival (Fig. 2). This NO-induced decreased survival was not affected by LAA co-administration and the cellular survival was further decreased with increased LAA concentration. These results indicated that high NO concentration is harmful to cellular survival.

Hydrogen peroxide-induced oxidative stress alse deoreased cellular survivial. When co-administered with LAA, the viability was increased significantly. These results indicated that LAA has a cytoprotective effect against oxidative stress through its well-known antioxidant effect.

4. Effect on NO production

LAA significantly potentiated NO production in a dose-dependent manner (Fig. 3). This enhanced NO production was inhibited by L-NAME, indicating that NO was produced by TM cells per se. Inhibition of NO production by L-NAME was decreased with increased LAA concentration. This finding also suggested that LAA enhances NO production in cultured TM cells. Sodium nitroprusside at 1 mM significantly increased NO production in the media and this increase was further potentiated by LAA. On the contrary LAA did not affect the NO production when co-administered with hydrogen peroxide (Fig. 4).

Discussion

Since the first report of significant amounts of ascorbic acid in the aqueous humor,[22] higher concentrations have been observed in many part of the eye.[23] In humans, ascorbic acid is the major water-soluble antioxidant.[24] It acts as a free

radical scavenger and may protect the lens against UV radiation under physiological conditions.[12,25]

Recently, several studies have shown that ascorbic acid potentiates endothelial NO synthesis in a dose- and time-dependent fashion,[13] and that it may protect NO from inactivation via chemical stabilization of tetrahydrobiopterin in endothelial cells.[26,27] Probably the two most important mechanisms of ascorbic acid are its abilities to protect NO by scavenging superoxide and peroxynitrite within endothelial cells, which enhances NO exit from the cells, and to spare NO synthase or tetrahydrobiopterin from oxidative modification.[14]

Because of their constant contact with the aqueous humor, TM cells are conceivably affected by the substances contained therein. Ascorbic acid is a significant component of the aqueous humor and its concentration is more than 10 times higher than that in plasma. Previous studies have demonstrated other possible roles of ascorbic acid, such as the ability to modify the synthesis of glycosaminoglycans[28] and to promote the production of extracellular matrix proteins, fibronectin, laminin[29] and collagen type I[30] by tissue-cultured, bovine TM cells. The high concentration of ascorbic acid in aqueous humor may affect the NO production and trabecular outflow since NO is a cellular mediator that can in particular modulate vascular tone as well as the aqueous humor dynamic in the eye.[31] There is some clinical evidence that ascorbic acid can enhance vasodilation due to NO donor. Altered levels of ascorbic acid in the aqueous humor of glaucomatous patients have been reported.[32,33] In addition, recent findings have demonstrated that NO can serve as an ocular hypotensive agent.[7,34-38]

Under the experimental conditions of this study, ascorbic acid potentiated NO synthesis in TM cells in a dose-dependent manner, which suggests that ascorbic acid may play an important role in maintaining trabecular outflow pathway by preserving NO. Oxidative stress induces the unavailability of tetrahydropterin and causes NO synthase to generate reactive oxygen substances. Excess superoxide will combine with NO to form peroxynitrite. This will result in both NO depletion and peroxynitrite-dependent oxidation of tetrahydropterin.[14]

Another possible mechanism by which ascorbic acid could preserve NO in tissues or plasma is to directly reduce nitrite to NO.[39] However, the extent to which this occurs in vivo is unknown. Nonetheless, since the nitrite is the major decomposition product of NO in aqueous solution, reduction of nitrite by ascorbic acid would effectively recycle and maintain local concentrations of NO.[40] Ascorbic acid was also known to reduce IOP by the depolymerization of the TM's hyaluronic acid component.[41]

Despite these in vitro findings, ascorbic acid concentration in aqueous humor did not affect surgical outcome[42] and clinical studies have demonstrated that the majority of open-angle glaucomatous eyes do not involve a deficiency of ascorbic acid and that ascorbic acid has no therapeutic value in the management of open-angle glaucoma.[32,43] This discrepancy may be due to the inability of ascorbic acid to induce NO production in already damaged or dysfunctional TM cells. To ascertain the role of NO, in vivo studies are required in addition to in vitro studies.

NO possesses a dual effect on cellular survival.[44,45] An excessive amount of NO is cytotoxic, while a smaller amount of NO can suppress cell death in different cell types.

Ascorbic acid inhibits proliferation of fibroblasts and retinal pigment epithelial cells.[46-48] In the present study, ascorbic acid did not affect cell viability, and the amount of NO produced by ascorbic acid was under the pathological concentration. However, sodium nitroprusside produced a large amount of NO and decreased the cellular survival. These findings suggest that ascorbic acid-induced NO plays an important physiological role in TM.

The tissues facing the anterior chamber of the eye are exposed constantly to aqueous containing reactive oxygen products generated by light-catalyzed reactions. Such radicals may cause increased oxidation in tissues of the anterior eye segment and finally lead to oxidative damage. Oxidative stress that exceeds the capacity of TM for detoxification could damage the TM cells with subsequent alteration of aqueous outflow resistance. Among these, hydrogen peroxide in aqueous humor is thought to originate from a light-catalyzed oxidation of ascorbic acid.[49] In cultured human TM, exposure to hydrogen peroxide resulted in considerable alteration in cellular morphology.[18,50] The present study demonstrated that ascorbic acid decreased TM cell death generated by hydrogen peroxide-induced oxidative stress and confirmed that ascorbic acid plays a cytoprotective role against oxidative stress.

Taking these results together, ascorbic acid might be involved in the maintenance of the TM function by enhancing NO production in this tissue, in addition to its role in the antioxidant function by removing reactive oxygen products such as hydrogen peroxide.

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