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The Role of Nitric Oxide in Ocular Surface Cells

The role of nitric oxide (NO) in the ocular surface remains unknown. We investigated the conditions leading to an increase of NO generation in tear and the main sources of NO in ocular surface tissue. We evaluated the dual action (cell survival or cell death) of NO depending on its amount. We measured the concentration of nitrite plus nitrate in the tears of ocular surface diseases and examined the main source of nitric oxide synthase (NOS). When cultured human corneal fibroblast were treated with NO producing donor with or without serum, the viabilities of cells was studied. We found that the main sources of NO in ocular surface tissue were corneal epithelium, fibroblast, endothelium, and inflammatory cells. Three forms of NOS (eNOS, bNOS, and iNOS) were expressed in experimentally induced inflammation. In the fibroblast culture system, the NO donor (SNAP, S-nitroso-N-acetyl-D, L-penicillamine) prevented the death of corneal fibroblast cells caused by serum deprivation in a dose dependent manner up to 500 μM SNAP, but a higher dose decreased cell viability. This study suggested that NO might act as a double-edged sword in ocular surface diseases depending on the degree of inflammation related with NO concentration.

Key Words : *Cell Survival; Nitric Oxide; Nitric-Oxide Synthase (NOS); Ocular Surface Tissue*

Jae Chan Kim, Gun Sic Park, Jin Kook Kim*, Young Myeong Kim†

Department of Ophthalmology, College of Medicine, Chung-Ang University, Seoul; Bal Geun Se Sang Clinic, Seoul; Department of Biochemistry†, College of Medicine, Kangwon National University, Chunchon, Korea

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Address for correspondence
Jae Chan Kim, M.D.
Department of Ophthalmology, College of Medicine, Chung-Ang University, Yongsan Hospital, 65-207 Hangangro-3-ga, Yongsan-gu, Seoul 140-757, Korea
Tel : +82.2-748-9580, Fax : +82.2-6491-9838
E-mail : jck50ey@kornet.net

INTRODUCTION

Nitric oxide (NO) is an unstable, short-lived, and potential toxic radical, produced by the oxidation of L-arginine by nitric oxide synthase (NOS) (1, 2). NO exhibits a rich biochemistry and a high reactivity and plays an important role as intercellular messenger in diverse physiologic processes, such as regulation of blood flow, neurotransmitter, and platelet aggregation (3).

NOS is divided into two major groups, constitutive NOS (cNOS) and inducible NOS (iNOS). cNOS are calcium-dependent and two isoforms are present, the brain form (bNOS) and the endothelial form (eNOS) (4, 5). In contrast, iNOS is a calcium-independent enzyme, and releases far greater amounts of NO and exerts cytotoxic and cytostatic effects not only against invading pathogens, but also against healthy cells (6-8). iNOS is induced in a number of cell types, such as fibroblasts, macrophages, and epithelium of the iris-ciliary body, especially after stimulation by cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and lipopolysaccharides (LPS) (9-12).

The effects of cell injury caused by NO have been postulated, both through direct or indirect chemical interactions with reactive oxygen species (ROS), such as superoxide to form other oxygen species that are potentially much toxic. Peroxynitrite is one of them, an extremely potent oxidant, which can lead to substantial cellular damage through lipid peroxidation and tyrosine nitration of protein (13, 14).

In the eye, it has been known that NO plays a significant role in the viability of cells in ocular surface tissue, but the role of NO in ocular surface diseases is still under investigation.

To assess the role of NO in ocular surface, we have firstly performed in vivo studies regarding the main source of NOS, the concentration of NO and NO-mediated toxicity in ocular surface diseases. Based on these in vivo experiments, finally, we have studied the critical NO level and its influence on cell survival with various concentrations of exogenous NO donor administered in corneal fibroblast culture system with or without serum.

MATERIALS AND METHODS

Nitric oxide assay, morphometric study, and western blotting (in vivo study)

All experiments were carried out in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Adult white rabbits (n=30), weighing 2-2.5 kg, were used in this study. The rabbits were divided into five groups. Group I was normal control. In group II, excimer laser ablation was performed using Apex-plus (Summit Technology, Waltham, MA, U.S.A.) (15). In group III, the experi-

mental allergic conjunctivitis was developed by topical application of codeine phosphate (mast cell activator) (16). Group IV was provoked by intravitreal injection of lipopolysaccharide (LPS) (10 ng/eye) (17). Group V was provoked by subconjunctival injection of NO donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP) (50 μM). Each group consisted of 6 rabbits. To investigate the effect of NOS inhibitors, each group was divided into two subgroups, and N-nitro-L-arginine methyl ester (L-NAME), 200 μg/eye and placebo Balanced salt solution (BSS) were injected subconjunctivally at 60 min before provocation as pretreatment.

The tear and aqueous humor samples of rabbits were collected using porous transorb polyester rod (American Filtrona Co. U.S.A.) and 30-gauge needle syringe, respectively. To evaluate the differences between human and rabbit, the tear samples of five healthy human volunteers (22-30 yr of age) were collected before and after excimer laser photokeratectomy as the same method. NO production was determined from stable end product of NO, nitrite plus nitrate concentration using Griess reaction (18).

The rabbits were killed by a large bolus injection of intravenous anesthetics. The cornea was obtained and bisected under the operating microscope. Bisected tissue sample was fixed in 4% paraformaldehyde and embedded in paraffin for staining with either toluidine blue for mast cell detection or, alternatively, with hematoxylin-eosin staining for routine histology and immunohistochemical study for eNOS, bNOS, iNOS (Transduction lab, U.S.A.) and TUNEL staining using apoptosis detection kit (Oncor, MD, U.S.A.). Another bisected sample embedded in O.C.T. compound (Sakura Finetek U.S.A., Torrance, CA, U.S.A.) for detection of α-nitrotyrosine formation was formed by powerful oxidant peroxynitrite, which indicated NO-mediated cellular damage.

We performed western blotting for detection of α-nitrotyrosine in photoablated corneal tissue. Corneal tissue was homogenized in boiling lysis buffer and centrifuged for 15 min. The supernatant was separated on 7.5% polyacrylamide gel electrophoresis and the proteins were blotted onto polyvinylidene difluoride membrane. The membrane was incubated with primary monoclonal anti-nitrotyrosine Ab (Upstate, NY, U.S.A.). The specific protein was detected with anti-rabbit IgG (Bio-Rad, CA, U.S.A.) conjugated with the horseradish peroxidase enzyme.

We also evaluated the proportion of apoptosis and necrosis using transmission electron microscopy.

Cell culture and cell viability assay (in vitro study)

Human corneal fibroblast was prepared from the remnant tissue of donor eyes after penetrating keratoplasty. The cells were grown and maintained in DMEM containing 10% fetal bovine serum, sodium bicarbonate, penicillin, and streptomycin of 100 units per mL. Fibroblasts were subcultured at 80-90% confluence for passage. The third passage of fibroblasts was seeded in 100 mm tissue culture dishes at a density of 10^6 cells. Fibroblasts were washed with serum-free DMEM medium and 2,500 cells were placed in each well in a 96-well tissue culture plate. Cells were divided into two groups (group I, serum-free media; group II, 10% fetal bovine serum-containing media). Each group was treated with different concentrations of NO donor SNAP (0, 1, 5, 10, 50, 100, 500, and 1,000 μM). After 24 hr, the reduction of 3-[4,5-cimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was used as an indicator of cellular toxicity. The assay was carried out in standard fashion using the cell proliferation kit 1 (MTT assay) (19). Results were given as optical densities (ODs) and were calculated as per cent of cell viability.

Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) and modified t-test to determine specific differences among groups and times.

RESULTS

Main sources of NO and peroxynitrite generation in ocular surface tissue

We found that the main sources of NO of ocular surface tissue were corneal epithelium, fibroblasts, endothelium, and

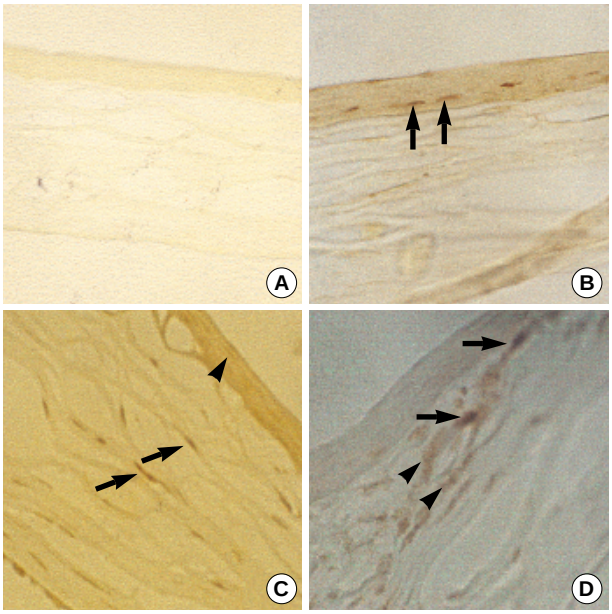

Fig. 1. A. Normal cornea (×100). B. Immunohistochemical localization of eNOS in epithelium of peripheral cornea (arrows) (×100). C. iNOS were strongly expressed in the corneal fibroblasts (arrows) and also expressed weakly in the corneal epithelium (arrow head) (×100). D. Increased iNOS expression in fibroblasts (arrow heads) and inflammatory cells (arrows) (×100).

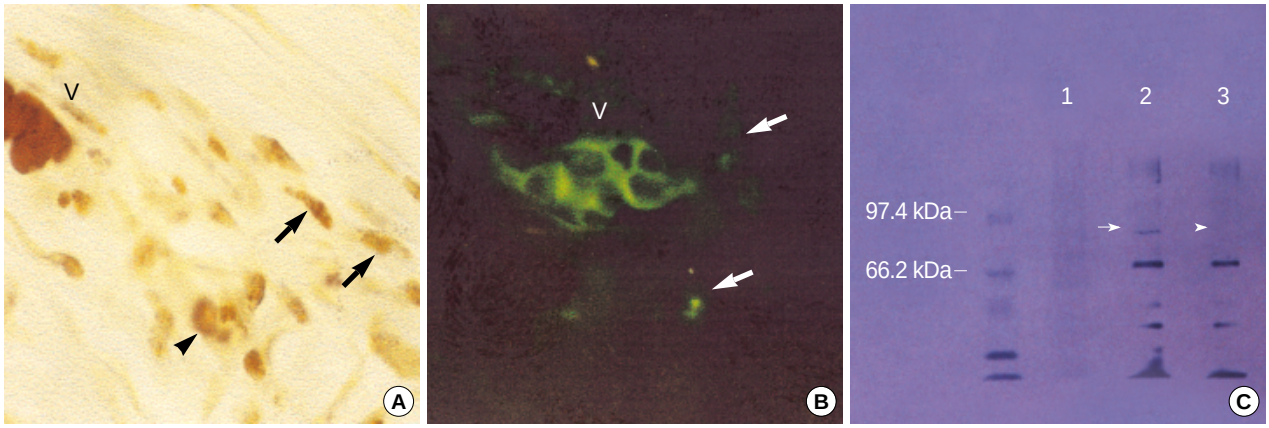

Fig. 2. A. The immunohistochemical localization of iNOS around the limbal vessel (V), fibroblast (arrow) and an inflammatory cell (arrowhead) (×400). B. α-nitrotyrosine staining around the limbal vessel (V) (×400). C. Lane 1, normal cornea. Lane 2, immunoblotting analysis of PTK-treated cornea reveals the overexpression of five nitrotyrosine-related proteins (arrow). Lane 3, PTK-treated cornea applied with L-NAME. The bands are weaker than those of lane 2 and the 97.4-kDa component disappeared (arrowhead).

inflammatory cells, which differently expressed the isoforms of NOS according to the inflammatory status. In normal control group, constitutive forms (eNOS and bNOS) were expressed negligible amount, revealed by immunohistochemical staining, and iNOS was absent. In contrast, all three forms of NOS were expressed in experimentally induced inflammation, such as photokeratectomy wound, LPS-induced uveitis and chemically induced allergic conjunctivitis. In photokeratectomy wound (Group II), eNOS was strongly expressed mainly in epithelium, and bNOS and iNOS were expressed mainly in fibroblasts. In addition, bNOS and iNOS were localized in inflammatory cells around limbal vessel 1 day after photokeratectomy (Fig. 1).

The localization of iNOS and α-nitrotyrosine was shown in Fig. 2A and B, depicting the staining patterns of inflammatory cells and limbal fibroblasts. In western blotting for α-nitrotyrosine, one labeled band (97.4 kDa), which indicated α-nitrotyrosine formation of protein caused by peroxynitrite, was shown in photoablated cornea and this band disappeared in NOS inhibitor (L-NAME)-treated group (Fig. 2C). In experimental allergic conjunctivitis, eNOS was expressed in subconjunctival vascular endothelial cells, and bNOS and iNOS were expressed in subconjunctival mast cells. As seen in the LPS-induced uveitis, iNOS was also expressed in many inflammatory cells and fibroblasts (data not shown).

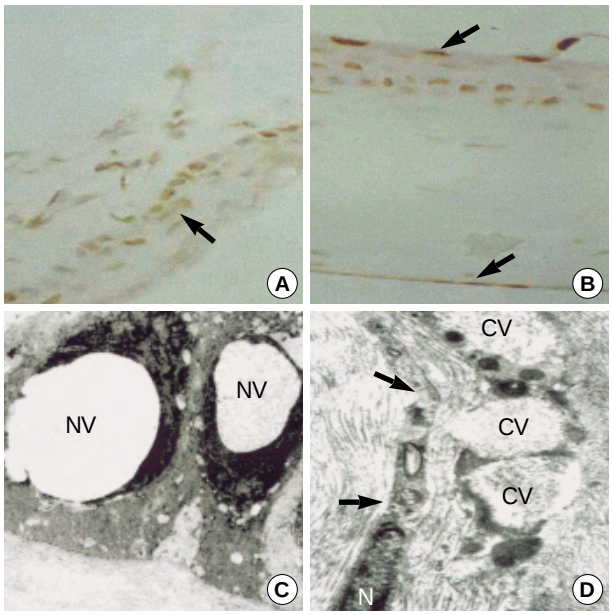

Fig. 3. A & B. The positive brown color for TUNEL staining cells (arrow) (×100). Specimen at 24 hr after LPS injection (A) or NO donor SNAP (B). C. Corneal epithelium at 24 hr after LPS injection (×4,000) ; perinuclear (NV) and cytoplasmic vacuolization. D. Fibroblasts at 24 hr after LPS injection (×4,000); the cytoplasm of fibroblasts are vacuolized (CV) and the chromatin is aggregated in the nucleus (N).

Critical NO concentrations and potential roles in tear and aqueous humor

Table 1A indicates NO concentrations in normal and various NO-related inflammatory conditions, such as photokeratectomy wound, allergic conjunctivitis, and LPS-induced uveitis. From the results of our experiment, we summarized the critical NO concentrations in Table 1B. The ratio 1.5-2.5 of nitric oxide value, assuming the normal nitric oxide value as 1, may play a defensive role defense, whereas much higher concentrations of NO (3 to 10 fold of nitric oxide value) may induce tissue damage.

NO-mediated toxicity in ocular surface

The corneal edema and sutural lens opacity were revealed

Table 1. NO concentrations and critical NO level

A. NO concentrations in tear and aqueous humor in normal and various pathologic conditions

	Tear (μM)	Aqueous humor (μM)
Normal	0.36±0.08†	4.12±0.95 μm*
	0.31±0.09*	
Photokeratectomy wound	0.73±0.07†	7.42±2.23 μm*
	0.62±0.12*	
Pathologic inflammation		
Experimental allergic conjunctivitis	2.31±0.59*	Not checked
LPS-induced inflammation	3.11±0.96*	18.32±3.64*
NO donor (SNAP)	3.71±1.84*	12.53±3.68*

* : rabbit, †: human.

B. Possible functional roles according to the critical NO level in tear and aqueous humor

	Normal	Normal wound healing (inflammation)	Pathological inflammation
Concentration ratio	1	1.5-2.5	3-10
Function	Physiologic	Defense mechanism Cell survival signaling Apoptosis	Tissue damage Oxidative stress Nitrosative stress DNA damage etc

Cell damage depends on the amount of NO and exposure time.

in LPS-induced uveitis (Group IV), which also occurred in NO donor-injected group (Group V). Corneal specimen showed remarkably increased edema in LPS-induced uveitis, while the edema was much decreased in NOS inhibitor (L-NAME)-treated group (data not shown). This corneal edema may indicate the loss of cellular function, which was caused by cell death.

The NO-mediated death of these cells was revealed by TUNEL-positive staining in corneal cells (Fig. 3A, B), which might indicate apoptosis. In electron microscopic findings, however, we observed that the NO-specific cell death was represented as atypical necrosis showing intranuclear or perinuclear vacuolization with nuclear compression, chromatin condensation and mitochondrial swelling (Fig. 3C, D). Thus the positive TUNEL staining may not be a specific finding indicating apoptosis.

Antiapoptotic actions of NO in serum-deprived corneal fibroblast

Since the main source of NO in corneal tissue was revealed to be fibroblasts, we examined whether NO have influence on apoptotic cell death of fibroblast in serum deprived condition.

In in vivo system, NO-mediated cell death is mainly shown as atypical necrosis with a few apoptosis. In contrast, in in vitro cell culture system, apoptotic cell death was easily observed

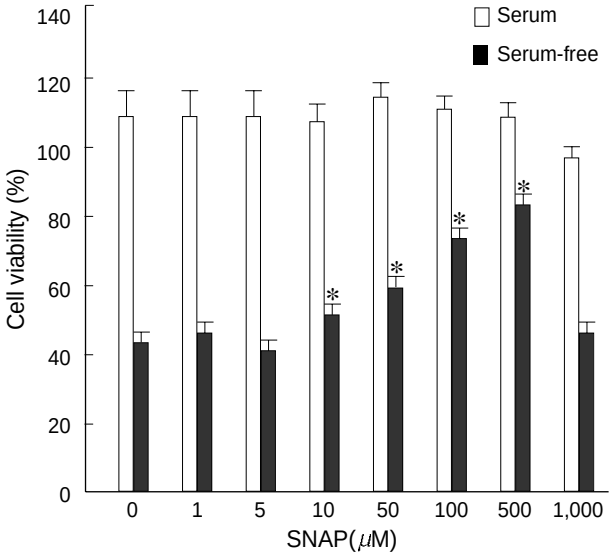

Fig. 4. Cytoprotective effects of NO donor (SNAP) on survival of corneal fibroblast in serum-deprived condition (*, p<0.05).

in the condition of serum-deprivation and high dose of NO donor, SNAP (data not shown). Serum-deprivation has also been known to induce apoptotic cell death in other types of fibroblast (20-22).

In serum-deprived fibroblast culture system, cell viability was increased in a dose-dependent manner from 10 μM to 500 μM NO donor (SNAP), but decreased at above 1,000 μM. NO did not affect the cell viability of serum-supplemented fibroblasts, but a higher dose (>1,000 μM) gradually decreased the cell viability (Fig. 4).

DISCUSSION

The present study shows that NO plays different pathophysiologic roles in the ocular surface depending on its concentration.

In the eye, it has been suggested that NO could participate in the pathogenesis of endotoxin induced uveitis (EIU) in rats (23-25), a model of certain types of human uveitis (26). In this context, we recently demonstrated that NO could be an important factor of the inflammatory mediators of corneal damage in streptozotocin-induced diabetic rats (27) and EIU in rats (28, 29) and also of the induction and progress of the allergic reaction to ocular surface (30).

NO seems to play an important role of physiological function, maintaining ocular surface cells, in tear and aqueous humor with its higher concentration being kept in aqueous humor than in tear, though some difference exists in each species of animals. Low concentrations of NO in the range of 0.3-1 μM in tear or 5-20 μM in aqueous humor may be involved in the process of wound healing after excimer laser ablation and defense mechanisms against allergic inflamma-

tion, whereas much higher concentrations of NO in the range of 1.5-5 μM in tear or 5-110 μM in aqueous humor may induce a significant corneal cell damage in LPS-induced uveitis and exogenous NO donor-injected group. Since the functional role was considered at this critical NO level in wound healing, the level of NO was implicated in the defense mechanism of maintaining cell survival signaling to avoid harmful inflammatory stress. In addition, a pathological NO level might serve as a toxicological function.

When inflammation was induced by excimer laser ablation and LPS injection, the expression of three forms of NOS was seen in the corneal cells, which was shown to be different according to the environment of the inflammation. When the amount of NO reached the maximum, the expression of iNOS was markedly increased in fibroblasts and inflammatory cells. It could be confirmed that the treatment of NOS inhibitor decreased the amount of NO and inflammation.

The tissue damage by NO could be confirmed by the formation of α-nitrotyrosine (13, 14). α-Nitrotyrosine was mainly expressed in inflammatory cells, especially localized in and around the limbal vessel. The expression of iNOS in limbal fibroblasts was likely to be related to some other functions, such as survival of limbal stem cell.

Because the main source of NO was fibroblast, we chose the fibroblast culture system to determine the role of NO in cell viability and critical NO level. We found that the NO donor (SNAP) prevented apoptosis of corneal fibroblasts caused by serum-deprivation in a dose-dependent manner up to 500 μM SNAP, while a higher dose decreased cell viability. Thus, these results suggested that NO-induced cytotoxicity worked biologically on the critical NO level in vitro as well as in vivo.

Therefore, NO may act as a double-edged sword in pathophysiological and toxicological mechanisms of ocular surface diseases, determined by its concentration and interaction with other oxygen mediators depending on the degree of inflammation.

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