Original Article

Effect and Mechanism of Phosphodiesterase Inhibitors on Trabecular Outflow

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Purpose: Phosphodiesterase (PDE) inhibitors increase matrix metalloproteinase (MMP) production by inhibiting re-uptake of adenosine and may potentiate nitric oxide (NO) activity. This study was performed to investigate the effects and mechanisms of PDE inhibitors on trabecular outflow in cultured human trabecular meshwork cells (HTMCs).

Methods: Primary HTMC cultures were exposed to 0, 20, and 50 µM dipyridamole (DPD) or theophylline (TPN). Permeability through the HTMC monolayer was assessed using carboxyfluorescein. The production of NO was assessed using the Griess assay and MMP-2 levels were measured via Western blotting.

Results: DPD significantly increased permeability accompanied with increased nitrite concentration and MMP-2 levels (all $p < 0.05$). TPN increased nitrite but did not affect permeability or MMP-2 levels significantly ($p > 0.05$). When treated with DPD and TPN together, both permeability and nitrite production were increased; however, MMP-2 levels showed no difference compared to DPD exposure alone ($p > 0.05$).

Conclusions: DPD increased trabecular permeability accompanied with increased nitrite production and MMP-2 levels. PDE inhibitors may increase trabecular outflow by increasing MMP-2 levels and by potentiating NO activity through cyclic GMP in HTMC.

Key Words: Dipyridamole, Matrix metalloproteinases, Nitric oxide, Theophylline, Trabecular meshwork

The trabecular meshwork (TM) of the eye, composed of cells and matrix, is thought to regulate aqueous humor outflow to control intraocular pressure (IOP). Among the factors controlling IOP, a pivotal role is ascribed to the TM, a smooth muscle-like tissue with contractile properties in the anterior chamber angle of the eye, that regulates aqueous humor outflow. It is thought that impaired drainage through the trabecular pathway caused by increased resistance is the primary cause of increased IOP in primary open-angle glaucoma [1,2].

While most glaucoma medications approved for clinical use act either on the uveoscleral pathway and/or aqueous humor formation, none target the major site of resistance to aqueous humor outflow in glaucoma. Therefore, novel pharmacological targets that can affect the conventional outflow pathway are highly desirable. Recently, several new drugs targeting the trabecular outflow pathway, including Rho-associated kinase inhibitors, adenosine agonists, and statins, have entered clinical development [3,4].

Among them, adenosine agonists increase trabecular outflow by changing TM cell volume, ion transport, and remodeling of the extracellular matrix (ECM) by increasing secretion of matrix metalloproteinase (MMP) [5-7].

Phosphodiesterase (PDE) inhibitors attenuate the nucleoside transporter responsible for the cellular uptake of adenosine and increase the secretion of MMP, similar to adenosine agonists. PDE inhibitors are also involved in cGMP hydrolysis. Therefore, PDE inhibitors could act by potentiating the action of mediators that work through cyclic GMP (cGMP), namely nitric oxide (NO) [8].

NO activates guanylate cyclase and generates cGMP and the cGMP signal is terminated by specific PDEs. The mechanism of activation of NO by PDE inhibitors is not yet understood. Previous study suggests that the effects of dipyridamole (DPD), a PDE inhibitor, on resistance vessels are preferentially explained by potentiation of adenosine mechanisms rather than potentiation of NO or other cGMP-mediated actions [8]. In addition, the PDE inhibitor theophylline (TPN) was shown to inhibit the production of NO [9], but another study revealed that DPD augments nitrite/NO production [10].

Although NO is known to increase trabecular outflow [11,12], the effect of PDE inhibitors on trabecular outflow and their action on the potentiation of NO is still unknown in the TM. This study was performed to evaluate the mechanism and effect of PDE inhibitors on trabecular outflow, particularly to determine the roles of MMP-2 and NO.

Materials and Methods

Cell culture and experimental treatment

This study followed the tenets of the Declaration of Helsinki and was approved by the institutional review board/ethics committee of Daegu Catholic University Hospital (CR-19-016-L). TM cell cultures were established from enucleated human eyes obtained from the eye bank. Briefly, TM tissues were excised by dissecting a continuous strand of tissue between the line of Schwalbe and the scleral spur by teasing away the TM tissue using a curette. The excised TM tissues were placed in a sterile culture dish with Dulbecco's modified Eagle media containing 15% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/mL gentamicin, 2.5 mg/mL fungizone, and left undisturbed for

three to five days at 37°C with a 5% CO_2 atmosphere. After identifying initial cell growth, the explants were removed, and the cultures were maintained with a medium containing 10% FBS. Cultures after three to five passages were used for experiments. The PDE inhibitors DPD or TPN (Sigma, St. Louis, MO, USA) were added to the cultures separately or simultaneously as described. In some experiments, 10 μM $N\omega$-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma), an endothelial nitric oxide synthase (eNOS) inhibitor, was added to the cultures to evaluate the de novo synthesis of NO.

Measurement of monolayer cell permeability with carboxyfluorescein

A permeability study of the TM cell monolayer was performed as previously described with minor modification [13-17]. Briefly, primary cultured human TM cells were incubated in the inner chamber (insert diameter, 12 mm; pore size, 0.4 μm) of a 12-well plate (no. 3460, Transwell; Corning, Lowell, MA, USA) at 2×10^4 cells/mL supplemented with 10% FBS. After the cells reached confluence, the media was changed to 1% serum-containing Dulbecco's modified Eagle media to avoid the effects of growth factors and proteins in serum. Then, the TM cells were exposed to each drug for 24 hours. After washing three times with PBS, 50 μM of the tracer carboxyfluorescein (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The media was collected from the outer well to analyze fluorescence after 2 hours, and the concentration of carboxyfluorescein in the collected media was measured using a spectrofluorometer (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

Measurement of NO production

Nitrite concentrations in the media were measured using the Griess reaction [18]. Briefly, media samples were collected from each well following appropriate treatment and reacted with modified Griess reagent (Sigma-Aldrich) by mixing equal volumes at room temperature for 15 minutes. Optical density was then measured and recorded 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.

Measurement of eNOS and MMP-2 mRNA expression levels

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). An RNA denaturation mix consisting of isolated RNA, oligo dT primers, and nuclease-free water was denatured. Reverse transcription polymerase chain reaction (RT-PCR) was performed using oligonucleotide primers specific to eNOS (forward primer, 5'-CTG GCT TTC CCT TCC AGT TC-3', 225 bp, reverse primer 5'-CCT TCC AGA TTA AGG CGG AC-3', 225 bp) or MMP-2 (forward primer, 5'-CTC GTG CCT TCCT AAG TCT GG-3', 251 bp, reverse primer, 5'-GGC GTT CCC ATA CTT CAC AC-3', 251 bp). Two reactions were run in parallel with a second reaction containing only Platinum Taq polymerase to assure that the source of the RT-PCR product was mRNA. cDNA was synthesized by adding prime RT premix, Taq Green Master Mix (Thermo Scientific, Carlsbad, CA, USA), and 10 pM of each forward and reverse primer. The amplification reaction was carried out for 30 cycles on a DNA Engine Cycler (Bio-Rad, Hercules, CA, USA). The amplified PCR products were analyzed using Multi-gauge software (Fujifilm, Tokyo, Japan) after electrophoresis. The level of b-actin was used as an internal standard.

Measurement of MMP-2 production by Western blotting

Cell extracts were prepared by lysing cells with RIPA buffer (Thermo Scientific). Samples were sonicated and cleared by centrifugation (1,200 rpm) at 4°C for 10 minutes. Supernatant protein concentrations were determined by BCA protein assay reagent (Thermo Scientific). Samples containing equal amounts of protein were separated by NuPAGE 4% to 12% Bis-Tris gels (Invitrogen), followed by transfer of resolved proteins to nitrocellulose membranes using the Xcell SureLock electrophoresis system (Invitrogen). Nonspecific binding was blocked for 1 hour at room temperature. Blots were then probed overnight at 4°C with primary antibodies, followed by 1-hour incubations with secondary antibodies conjugated to goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA), and then developed by chemiluminescence detection using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Quantification of the signals was performed using a Gel Doc XR+ system (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal standard.

Statistical analysis

All data represent the average results of at least three independent experiments. Experimental differences between the results of control cultures and a single treatment group were evaluated using Student's t-tests. A p-value less than 0.05 was considered statistically significant.

Results

Effects of PDE inhibitors on the permeability of trabecular cell monolayers

Administration of 20 or 50 μM DPD to human trabecular meshwork cell monolayers significantly increased the concentration of carboxyfluorescein in the outer well (permeability) compared to non-exposed controls ($p = 0.004$, 0.035). Administration of TPN showed a tendency to increase permeability but the trend was not statistically significant ($p = 0.227$, 0.099). Co-exposing monolayers to both 20 μM DPD and TPN, or 50 μM DPD and TPN increased permeability compared to non-exposed controls ($p = 0.001$, 0.001) (Fig. 1).

Effects of PDE inhibitors on NO production

Administration of either 20 or 50 μM DPD or TPN significantly increased nitrite concentration in the media respectively (all $p < 0.05$). Co-exposure to 20 μM DPD and TPN, or 50 μM DPD and TPN increased nitrite concentration compared to non-exposed controls, respectively (all $p < 0.05$) (Fig. 2).

Effects of PDE inhibitors on eNOS and MMP-2 mRNA expression

In order to determine if the increased nitrite concentrations by DPD were caused by de novo synthesis of NO, the levels of eNOS mRNA expression were measured. As a re-
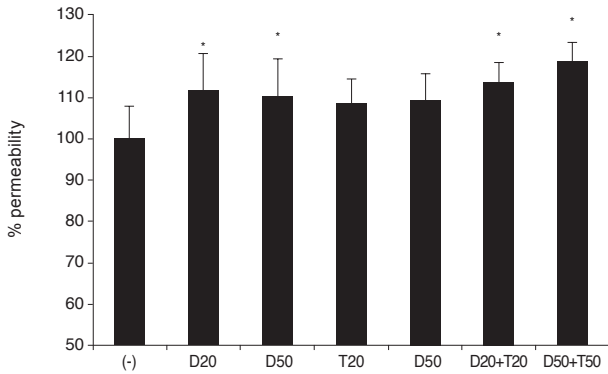
Fig. 1. Effect of phosphodiesterase inhibitors on permeability through the trabecular cell monolayer. Exposure to 20 or 50 μM dipyridamole (D) or theophylline (T) significantly increased the concentration of carboxyfluorescein in the outer well (permeability) compared to the non-exposed control (-). Co-exposure to 20 μM D and T, or 50 μM D and T further increased permeability. Carboxyfluorescein intensity of the outer chamber was normalized to the mean value obtained using a non-exposed control (permeability 100%, $^*p < 0.05$).
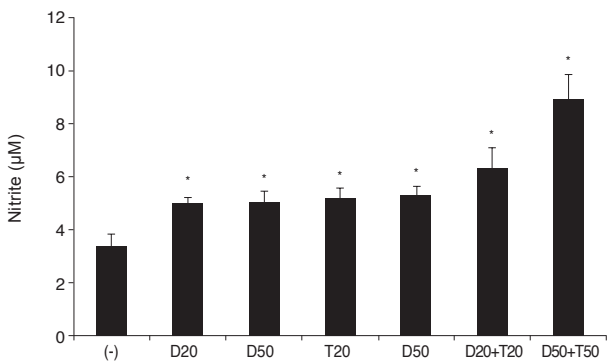

Fig. 2. Effect of phosphodiesterase inhibitors on the production of nitric oxide. Exposure to 20 or 50 μM dipyridamole (D) or theophylline (T) increased the concentration of nitrite significantly compared to the non-exposed control (-). Co-exposure to 20 μM D and T or 50 μM D and T further increased the concentration of nitrite ($^*p < 0.05$).
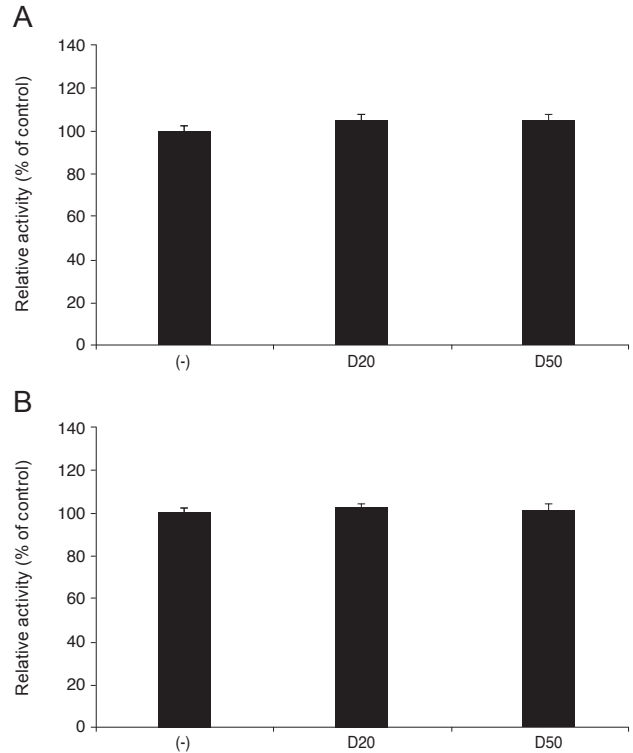

Fig. 3. Effect of dipyridamole (D) on the expression of (A) endothelial nitric oxide synthase mRNA and (B) matrix metalloproteinase 2 mRNA. Exposure to 20 or 50 μM dipyridamole did not significantly affect the expression of endothelial nitric oxide synthase mRNA or matrix metalloproteinase 2 mRNA compared to non-exposed control (-) ($p > 0.05$).

sult, neither 20 nor 50 μM DPD affected the levels of eNOS mRNA expression compared to non-exposed controls ($p = 0.088$, 0.062) (Fig. 3A). In addition, the levels of MMP-2 mRNA expression were also measured to determine the involvement of transcription on MMP-2 levels. As a result, DPD at both 20 and 50 μM concentrations did not affect the levels of MMP-2 mRNA expression compared to non-exposed controls ($p = 0.148$, 0.628) (Fig. 3B).

Effects of PDE inhibitors on MMP-2 levels

Administration of 50 μM DPD significantly increased

MMP-2 levels compared to non-exposed controls ($p = 0.018$). Co-exposure to 20 μM DPD and TPN, or 50 μM DPD and TPN increased MMP-2 levels compared to non-exposed controls ($p = 0.041$, 0.031). When comparing 20 μM DPD and TPN co-exposure with exposure to 20 μM DPD alone, the MMP-2 levels did not differ ($p = 0.130$). When co-exposure to 50 μM DPD and TPN was compared to exposure to 50 μM DPD alone, the MMP-2 levels did not significantly differ ($p = 0.309$) (Fig. 4). These results suggest DPD had a stronger effect on MMP-2 than TPN. To test this, 20 μM or 50 μM TPN alone were used assess MMP-2 levels. As a result, TPN did not significantly increase MMP-2 levels compared to non-exposed controls (Fig. 5).

Effect of eNOS inhibitors on NO production and permeability

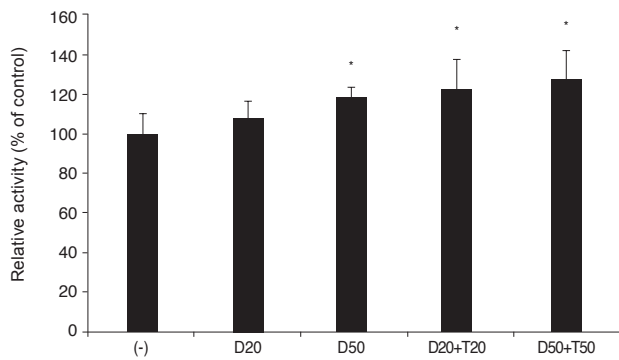Administration of 10 μM L-NAME decreased nitrite

Fig. 4. Effect of phosphodiesterase inhibitors on the activity of matrix metalloproteinase (MMP)-2 levels measured by Western blotting. Exposure to 50 µM dipyridamole (D) significantly increased MMP-2 activity compared to non-exposed controls (-). Co-exposure to 20 µM D and theophylline (T), or 50 µM D and T further increased MMP-2 production ($^*p < 0.05$).
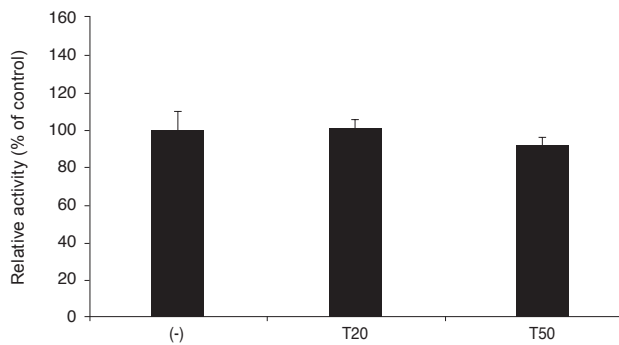

Fig. 5. Effect of theophylline (T) on the matrix metalloproteinase 2 levels measured by Western blotting. Exposure to either 20 or 50 µM T did not significantly affect the matrix metalloproteinase 2 levels compared to non-exposed controls (-).

A

B

Fig. 6. Effect of endothelial nitric oxide synthase inhibitor on the production of (A) nitric oxide and (B) permeability of trabecular meshwork cell monolayer. Exposure to 20 µM dipyridamole (D) with or without 10 µM N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, L) increased the production of (A) nitrite and (B) permeability compared to non-exposed controls (-) ($^*p < 0.05$). Co-exposure to 20 µM D and 10 µM L-NAME compared to exposure to 20 µM D alone, the production of nitrite and permeability showed no statistically significant differences ($p = 0.864, 0.815$).

production compared to non-exposed controls ($p = 0.045$). Exposure to 20 µM DPD or co-exposure to 20 µM DPD and 10 µM L-NAME increased nitrite production compared to non-exposed controls ($p = 0.010, 0.017$). When comparing to 20 µM DPD and 10 µM L-NAME co-exposure with exposure to 20 µM DPD alone, the concentration of nitrite showed no statistically significant difference ($p = 0.864$) (Fig. 6A). In addition, exposure to 20 µM DPD or co-exposure to 20 µM DPD and 10 µM L-NAME increased permeability compared to non-exposed controls ($p = 0.024, 0.048$). Co-exposure to 20 µM DPD and 10 µM L-NAME compared to 20 µM DPD alone resulted in no statistically significant difference in permeability ($p = 0.815$) (Fig. 6B). As described above, PDE inhibitors did not affect eNOS mRNA expression, suggesting that the ef-
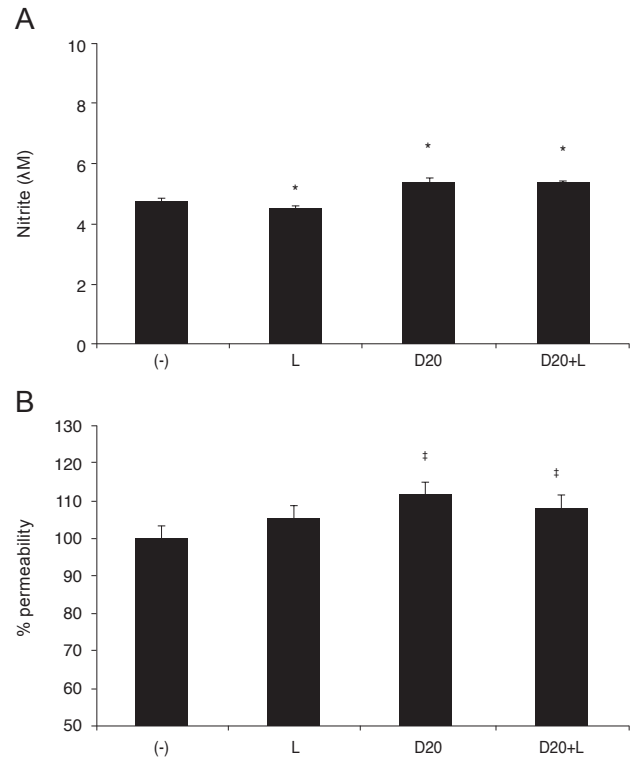
fect of PDE inhibitors on NO was caused by potentiation of NO activity, and not by de novo synthesis of NO.

Discussion

Adenosine is a ubiquitous local modulator that regulates various physiological and pathological functions by stimulating membrane receptors. There is substantial evidence that functional adenosine receptors (ARs) are expressed in the cells of the outflow pathway [19] and their activity has been shown to exert significant effects in aqueous outflow facility and IOP [20-23].

Adenosine and several adenosine derivatives have been shown to increase and/or decrease IOP. The effects of ade-

nosine agonists or antagonists are species dependent. In general, adenosine binds to A1 AR of the TM to reduce outflow resistance, and thereby lowers IOP. Activation of A2A AR can alter outflow resistance at the Schlemm's canal cell level, and thereby increase and/or decrease IOP. Adenosine stimulates an A3 AR-mediated increase of Cl− channels of the non-pigmented epithelial cells of the ciliary epithelium and aqueous humor fluid inflow, thereby increasing IOP, while A3 AR antagonists prevent adenosine-induced activation of non-pigmented epithelial cell Cl− channels of the ciliary epithelium, and thereby lower IOP [5].

Resistance to conventional aqueous outflow is in part dependent on the composition of the ECM in the TM. Human trabecular meshwork cells may regulate outflow resistance by modifying their surrounding ECM through the secretion of MMPs. Adenosine A1 ARs have been shown to decrease outflow resistance by increasing MMP-2 secretion [24,25]. Since PDE inhibitors increase adenosine concentration by attenuating the uptake of adenosine, it could increase outflow facility by increasing MMP-2 secretion. Thus, there is a possibility that PDE inhibitors such as AR antagonists increase trabecular outflow by increasing MMP-2 secretion like adenosine. This study revealed that the PDE inhibitor DPD increased the TM cell monolayer permeability accompanied with increasing secretion of MMP-2. Since DPD did not increase MMP-2 mRNA, the increased activity of MMP-2 can be explained by increased translation or stabilization of post-transcription modifications rather than by changing transcriptional events.

Potentiation of NO activity was suggested as another mechanism of action of PDE inhibitors, but previous studies about the effects of PDE inhibitors on NO activity have yielded controversial results [8-10,26]. In this study, both DPD and TPN increased nitrite in the media and additional treatment of TPN to DPD further increased nitrite. These findings suggest that PDE inhibitor mechanisms of action are explained by potentiation of NO activity in addition to potentiation of the adenosine mechanism by increasing MMP-2 levels.

The TPN PDE inhibitor did not affect the permeability or MMP-2 levels in contrast to DPD. Possible explanations about this discrepancy between DPD and TPN might be explained as follows. The xanthine derivative TPN is a relatively weak non-selective inhibitor of PDE isoenzymes

and also has other diverse mechanisms of action [5,27]. As TPN did not decrease permeability, there is a possibility that TPN may act primarily on the A1 and A2 AR rather than on the A3 AR, which is known to increase IOP. Although TPN alone did not increase permeability, the permeability was further increased when added to DPD. Thus, it is possible that TPN could potentiate NO activity. The molecular mechanism of TPN action is still not completely understood. Further study may be needed to explore the mechanisms of TPN in the future.

PDE inhibitor-induced increased nitrite production can be generated either by increased de novo synthesis of NO or by potentiation of NO activity. Adding L-NAME did not affect the degree of permeability, nitrite production, or eNOS mRNA expression in this study. Thus, PDE inhibitors potentiate NO activity by attenuating degradation of cGMP rather than increasing de novo synthesis of NO.

Previous studies suggested that NO could act as a negative regulator in cellular migration by down-regulating MMP-2 expression [28,29] but these studies used NO donors or eNOS activators to stimulate NO production. Our study demonstrates that PDE inhibitors do not suppress MMP-2 secretion despite increased NO activity. These results support that PDE inhibitors potentiate NO activity through cGMP. NO is well known to activate intracellular guanylyl cyclase to produce cGMP. Taken together, increased NO activity exerts its effects through increasing cGMP and results in increased permeability.

The interaction between NO and adenosine was suggested as another postulated mechanism of action for DPD. NO inhibits protein kinase C, which activates 5'-nucleotidase, the enzyme responsible for the generation of adenosine from 5'-adenosine monophosphate [8]. Thus, NO activity may be potentiated by not only cGMP and but also cAMP, and both result in dilatation of the TM.

Numerous MMPs and their inhibitors are expressed in the TM [30-32]. Among them, MMP-2 was constitutively expressed at relatively high levels but MMP-9 was expressed at low levels in the TM [30]. The effect of adenosine agonists on MMP-9 secretion is unclear [6,25,33-35]. Previous study showed that DPD decreased MMP-9 secretion [33]. Although the activity of other MMPs were not evaluated in this study, DPD increases MMP-2 activity similar to adenosine agonists. Future research should evaluate the specific roles for the different MMPs.

In conclusion, DPD increased trabecular permeability

accompanied with both increased MMP-2 levels and NO activity. Thus, PDE inhibitors potentiate endogenous adenosine action by blocking cellular uptake (by increasing MMP-2) and may potentiate NO activity by inhibiting cGMP-specific PDE.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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