Effects of Protein Kinase Inhibitor, HA1077, on Intraocular Pressure and Outflow Facility in Rabbit Eyes

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Objective: To elucidate the roles of protein kinase in regulating the intraocular pressure (IOP) and outflow facility in rabbit eyes.

Materials and Methods: A protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-homopiperazine (HA1077), was used. The IOP and the outflow facility were measured before and after topical, intracameral, or intravitreal administration of HA1077 in rabbits. Western blot analysis was performed to detect the 20-kd light chain of myosin in human trabecular meshwork (TM) cells and bovine ciliary muscle (CM) tissues. The cell morphologic condition and distribution of actin filaments and vinculin in TM cells were studied using cell biology techniques. Carbachol-induced contraction of isolated bovine CM strips following administration of HA1077 was examined in a perfusion chamber.

Results: In rabbit eyes, the administration of HA1077 resulted in a significant decrease in IOP in a dose-dependent manner. An increased outflow facility was also observed. Western blot analysis revealed the presence of 20-kd light chain of myosin in human TM cells and bovine CM tissues. In cultured human TM cells, exposure to HA1077 disrupted actin bundles and impaired focal adhesion formation. In addition HA1077 showed relaxation of bovine CM strips.

Conclusions: Use of HA1077 caused a reduction in IOP and an increase in the outflow facility. The results of in vitro experiments suggest that the IOP-lowering effects of HA1077 may be related to the altered cellular behavior of TM cells and relaxation of CM contraction. The results of these studies suggested that protein kinase inhibitors have the potential to be developed into a treatment modality for glaucoma.


In Glaucomatous eyes, elevation of intraocular pressure (IOP) is believed to be one of the major factors that causes axonal damage in the optic nerve head and the subsequent retinal ganglion cell death, leading to blindness.1,2 The IOP is regulated essentially through 2 routes of the aqueous humor outflow—conventional (trabecular) and unconventional (uveoscleral) pathways.3 Conventional outflow, the major pathway, is influenced by the cellular behaviors and cell-cell junctions of trabecular meshwork (TM) cells.3 In the TM system, series of investigations have indicated that the alterations in the contractility and cellular behaviors of TM cells can affect the IOP and the aqueous outflow.3,5-9

An antivasospastic compound, 1-(5-isoquinolinesulfonyl)-homopiperazine (HA1077), has been previously shown to act as a vasodilator in vivo and is currently used for the treatment of cerebral vasospasm, inhibiting agonist-induced smooth muscle contraction.10 This compound has also been shown to be able to induce inhibition of smooth muscle contraction and alter various cellular behaviors.11,12

Rho GTPase, a member of the Rho subgroups of the Ras superfamily, is involved in diverse physiological functions associated with cytoskeletal rearrangements,13,14 such as cell morphology,15 cell motility,16 cytokinesis,17 and smooth muscle contraction.18,19 Recently, several putative target molecules of the Rho have been identified as Rho effectors, including p160ROCK,20,21 ROKalpha/Rho kinase/ROCK II,22-24 and protein kinase N (PKN).20 ROCK has been shown to phosphorylate the largest subunit of myosin phosphatase in the carboxyl terminal region, resulting in inhibition of the phosphatase activity.25,26 This inhibition is suggested to be responsible for the Rho-mediated Ca++ sensitization process.27,28 The ROCK-mediated inhibition of myosin phosphatase also accounts for an

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MATERIALS AND METHODS

ANIMALS AND ANESTHESIA

Adult Japanese white rabbits, weighing 2 to 2.5 kg, were used in this study. All studies were conducted in accord with the Association of Vision Research in Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. For IOP measurements, the rabbit eyes were anesthetized by topical instillation of 2% lidocaine hydrochloride. For measurements of the outflow facility or the uveoscleral outflow, the rabbits were anesthetized with 40% urethane (1.0-1.5 mL/kg).

CHEMICALS, DRUG PREPARATION, AND DRUG ADMINISTRATION

The HA1077 was supplied by Asahi Chemical Industry Co Ltd., Tokyo, Japan. Carbachol (CCh); fluorescein isothiocyanate conjugated–phalloidin, mouse monoclonal antibody to vinculin, and MLC were obtained from Sigma Chemical Co., St Louis, Mo. Appropriate secondary antibodies were obtained from Chemicon International, Temecula, Calif. For topical administration, HA1077 (1 or 10 mmol/L) was administered as four 3-µL drops, to give a 1- or 10-µmol/L concentration in the approximately 120-µL rabbit anterior chamber33 assuming 1% intracameral penetration and no drug loss from the anterior chamber.34,35 After topical anesthesia of rabbit eyes, 1.2 µL of 0.1-, 1-, or 10-µmol/L HA1077 was administered intracamerally (1-, 10-, or 100-µmol/L final concentration, respectively) and 14 µL of 1- or 10-µmol/L HA1077 was administered intravitreally (10- and 100-µmol/L final concentration, respectively) in the approximately 1.4-mL vitreous space in the rabbit eye.33 The fellow eyes that were treated with vehicle phosphate-buffered solution served as controls.

RESULTS

IOP MEASUREMENT AND SLITLAMP BIOMICROSCOPY

Compared with contralateral vehicle-treated controls, the IOP in rabbit eyes was significantly (P<.01) lowered at 0.5 hour following topical administration of a 10-µmol/L concentration of HA1077 eyedrops. The IOP reduction was maximally observed at 3 hours with a 10-µmol/L concentration of HA1077 (Figure 1A). After intravitreal administration, significant IOP reductions were noted between 0.5 and 12 hours, and the maximal reductions were seen between 3 and 6 hours with the 100-µmol/L concentration (P<.001) (Figure 1B). When administered intracameraly, significant IOP reductions occurred between 0.5 and 12 hours and 100-µmol/L HA1077 produced the maximal reductions (P<.001) (Figure 1C). No anterior chamber, lens, or fundus abnormalities in rabbit eyes were detected by slitlamp examination following either the topical, intracameral, or intravitreal administration of HA1077. These experiments, thus, demonstrated the potent IOP-lowering effects of HA1077 in rabbit eyes.

MEASUREMENTS OF THE OUTFLOW FACILITY

The outflow facility was measured 3 hours after topical administration of HA1077 when maximal IOP reduction was observed. Results summarized in the Table...
TM cells from passages 3 through 8 were used for subsequent studies.

PREPARATION OF LYSATES FROM WHOLE CELL AND BOVINE TISSUE AND IMMUNOBLOTTING

To examine the expression of MLC, detergent lysates of TM cells and bovine CM tissue were prepared in Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Co, Bedford, Mass) and incubated serially with primary and secondary antibodies. The blotted protein bands were visualized with an immunostain (Konica immunostain HRP-1000; Konica, Tokyo, Japan).

EFFECTS OF HA1077 ON CELL SHAPE OF HUMAN TM CELLS

In experiments designed to examine changes in cell shape, postconfluent and semiconfluent TM cultures were incubated with various concentrations of HA1077 (1-1000 µmol/L) with or without serum. The cultures were observed by phase-contrast microscopy and photographed immediately after drug application, and 10, 30, and 60 minutes later. The drug solution was removed afterward and replaced with plain Dulbecco Eagle minimum essential medium containing 10% FBS. In all cases, recovery of normal morphology was documented 2 and 15 hours later.

ACTIN AND VINCULIN STAINING

Human TM cells were plated on coverglasses at a density of 3 x 10^4 cells per each 6-cm dish. After culturing for 2 days, when cells reached semiconfluence, HA1077 was added and incubated. For controls, phosphate-buffered saline solution was added as a vehicle. After the drug exposure, the cells on coverglasses were fixed with 3% paraformaldehyde–phosphate-buffered saline solution and 0.3% Triton X-100 (Sigma Chemical Co) for 20 minutes. Filamentous actin (F-actin) was labeled with fluorescein isothiocyanate conjugated–phalloidin (0.05 mg/mL) for 1 hour. For vinculin staining, the coverglasses were incubated successively with antivinculin antibody (1:400) for 1 hour and with secondary antibody for 30 minutes. Fluorescence was visualized under an epifluorescence microscope (Zeiss Axioplan, Oberko-chen, Germany) and with a confocal laser scanning microscope (Bio-Rad, Hercules, Calif). To determine whether the effects of HA1077 were reversible, the cells were incubated for another 30 minutes in HA1077-free medium after the various HA1077 treatments, fixed, and stained.

MEASUREMENT OF CONTRACTILITY OF CM

Enucleated bovine eyes were obtained from a local slaughterhouse and placed on ice. Small bovine CM strips were carefully dissected according to procedures described by Lepple-Wienhues et al.4 Briefly, after excision of the iris, meridional CM strips were excised. The CM contractility was measured isometrically with a force-length transducer device using an isometric force transducer connecting an amplifier, a multipen recorder, and vertically mounted in a 20-ml Magnus tube filled with continuously aerated Krebs-Hensleit solution. Only CM strips that showed a stable tone were used for experiments. The HA1077 was added cumulatively to the bath. Relaxation responses were expressed as a percentage of the maximum effect (100%) elicited by CCh in each strip.

STATISTICAL ANALYSIS

Data were analyzed by repeated measured analysis of variance and Bonferroni adjustment as a post hoc test of time course of IOP. Mann-Whitney test was used for aqueous humor dynamics. P < .05 was considered to be statistically significant.

EFFECTS OF HA1077 ON MORPHOLOGY OF CULTURED HUMAN TM CELLS

Next, the morphology of TM cells was examined. By phase-contrast microscopy, treatment with 100-µmol/L HA1077 in the presence of serum for 30 minutes induced retraction and rounding of TM cells (Figure 3). When semiconfluent cultures were treated with HA1077, TM cells also retracted and became thinner (Figure 4B, upper 4 rows). To determine whether such changes were related to the Rho/ROCK pathway by serum stimulation, the cells were also incubated in serum-free medium. Retraction and thinning were seen 30 to 60 minutes later (Figure 4B, bottom row). These results showed that the TM morphology might be influenced by inhibition of the Rho/ROCK signaling system.

EFFECTS OF HA1077 ON CYTOSKELETON OF CULTURED HUMAN TM CELLS

To examine whether the actin structure was affected, 1-, 10-, 100-, or 1000-µmol/L HA1077 was added to the culture medium. It was found that the distribution of F-
actin was altered dramatically in a time- and concentration-dependent manner (Figure 5). In control cells, actin filaments were assembled into large radial and circumferential bundles in association with focal adhesions (Figure 5A). As Figure 5B shows, HA1077 produced distinctive effects on the microfilament organization in TM cells. Treatment with 100-µmol/L HA1077 for 30 minutes caused loss of most of their actin bundles in TM cells (Figure 5B). After treatment with 10-µmol/L HA1077 for 60 minutes, the stress fibers in the center of TM cells were labeled with phalloidin; however, the peripheral bundles were lost. Vinculin in control cells was predominantly associated with focal adhesions (Figure 5A). After the HA1077 treatment, deterioration of focal adhesions in the cell periphery was evident (Figure 5B). These cytoskeletal changes were reversible within 2 hours, and completely recovered after 15 hours.

**MEASUREMENT OF CONTRACTILITY OF ISOLATED CM**

After adjustment of baseline tension, CCh at a concentration of 10^{-6} mol/L was used to induce contraction in
isolated bovine CM strips as described previously.\textsuperscript{42,43}

**Figure 6** shows a typical recording of the relaxation effects induced by cumulatively added HA1077. Superfusion by the CCh resulted in an immediate steep force development that reached maximum after 3 minutes. The HA1077 led to relaxation of the CCh precontracted bovine

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Observation</th>
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<tbody>
<tr>
<td>1 µmol/L</td>
<td>Retraction and thinning of the cells (arrows)</td>
</tr>
<tr>
<td>10 µmol/L</td>
<td>Serum starvation also resulted in a similar cell shape change with retraction and thinning of the cells (arrow)</td>
</tr>
<tr>
<td>100 µmol/L</td>
<td>The drug solution or Dulbecco Eagle minimum essential medium without serum was removed afterward and replaced with Dulbecco Eagle minimum essential medium containing 10% fetal bovine serum. Recovery of normal morphologic status was observed 2 and 15 hours later (original magnification ×60).</td>
</tr>
<tr>
<td>Serum(−)</td>
<td>Serum starvation also resulted in a similar cell shape change with retraction and thinning of the cells (arrow)</td>
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*Figure 4. Effects of HA1077 on morphology of cultured human trabecular meshwork (TM) cells. Phase-contrast microscopic observation of semiconfluent culture of human TM cells. Treatments with HA1077 in concentrations of 1 µmol/L (upper row), 10 µmol/L (upper middle row), 100 µmol/L (middle row), and 1000 µmol/L (lower middle row) for 10, 30, and 60 minutes in the presence of serum resulted in retraction and thinning of the cells (arrows). Serum starvation also resulted in a similar cell shape change with retraction and thinning of the cells (lower row; arrow). The drug solution or Dulbecco Eagle minimum essential medium without serum was removed afterward and replaced with Dulbecco Eagle minimum essential medium containing 10% fetal bovine serum. Recovery of normal morphologic status was observed 2 and 15 hours later (original magnification ×60).*
Figure 5. Distribution of filamentous actin (F-actin) and vinculin in human trabecular meshwork (TM) cells treated with HA1077. A, Distribution of F-actin (in green) and vinculin (in red) in normal human TM cells. Small white arrows show F-actin bundles, and white arrowheads show focal adhesions associated with vinculin. a, Confocal images. b, Cells were stained with antibody to vinculin. c, Cells were stained with fluorescein isothiocyanate conjugated–phalloidin to visualize F-actin. B, Distribution of F-actin and vinculin in human TM cells treated with HA1077 in concentrations of 1, 10, 100, and 1000 µmol/L for 10, 30, and 60 minutes. The drug solutions were removed afterward and replaced with Dulbecco Eagle minimum essential medium containing 10% fetal bovine serum. Recovery of normal morphologic status was observed 2 and 15 hours later. White arrows point to F-actin bundles, which disappeared with HA1077 treatment and recovered after drug removal. White arrowheads show vinculin-containing focal adhesions that were decreased with HA1077 treatment and recovered by replacement with Dulbecco Eagle minimum essential medium. Bar indicates 10 µm.
CM strips in a dose-dependent manner. The maximum effect was found in experiments using $10^{-3}$-mol/L HA1077, which almost completely abolished (by 99%) the response to CCh. Recovery of contractility in the presence of CCh after the removal of HA1077 was also observed. Figure 7 shows the data obtained with increasing concentrations of HA1077. At $10^{-3}$- to $10^{-5}$-mol/L concentrations, the HA1077-induced relaxation of the CCh precontracted bovine CM strips was significant. The average ± SD relaxation for HA1077-treated bovine CM strips was 6.5% ± 1.0%, 10.8% ± 1.1%, 28.5% ± 5.8%, 42.5% ± 2.6%, and 99.3% ± 0.5%, for $10^{-7}$-, $10^{-6}$-, $10^{-5}$-, $10^{-4}$-, and $10^{-3}$-mol/L HA1077, with $P$ values being .33, .78, .02, .001, and <.001, respectively, compared with the time-matched controls.

**COMMENT**

The present study demonstrated that a protein kinase inhibitor, HA1077, when administered topically, intracameral, or intravitreally, induces a significant decrease in IOP in rabbit eyes. To elucidate the mechanisms of the IOP-lowering effects of this inhibitor, we have conducted a series of experiments.

First, physiologic experiments showed that HA1077 elicits changes in the total outflow facility, but not in the unconventional one. Conventional outflow is the main route in human and primate eyes, whereas, or intravitreally, induces a significant decrease in IOP in rabbit eyes. To elucidate the mechanisms of the IOP-lowering effects of this inhibitor, we have conducted a series of experiments.

Second, our cytochemical studies demonstrated that HA1077 disrupted F-actin bundles and impaired focal adhesion formation in the cultured TM cells. Similar findings have been reported previously on the serine-threonine–kinase inhibitor, H-7, which also disrupts cell junctions and results in a decrease in IOP.

Light chain of myosin phosphorylation is known to be a mechanism that controls the actomyosin contractility in many cell lines and is reported to be essential and sufficient for the formation of stress fibers and focal adhesions in fibroblastic cells. It has been previously shown that HA1077 inhibits Rho-mediated enhancement of Ca$^{2+}$-induced MLC phosphorylation. It was also demonstrated previously that HA1077 inhibits not only ROCK but also PKN, another Rho-associated protein kinase. However, it was demonstrated that in vitro PKN neither phosphorylated myosin phosphatase nor inhibited its activity. These observations are consistent with the notion that the effects of HA1077 on TM cell integrity may be related to cytoskeletal changes induced by the alteration in balance of MLC phosphorylation mediated by ROCK. Our immunoblot results showed that MLC is present in cultured human TM cells; this also supports our hypothesis.

Furthermore, in our experiments using bovine CM strips, HA1077 led to relaxation of the smooth muscle in a dose-dependent manner. Many investigators reported that relaxation of CM would not increase trabecular outflow. Although similar findings have been reported in previous studies, we are unable to conclude that HA1077-induced changes in CM contribute to the hypotensive effects of this drug. Further studies will be required to assess the role of CM relaxation in the IOP-lowering effects of HA1077.

In summary, this study shows that HA1077, a protein kinase inhibitor, reduces IOP and increases outflow facility. Such effects may be related to altered cellular behavior of TM cells. Inhibition of the Rho signaling
pathway may be developed into a new strategy for the treatment of glaucoma.

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