Effect of Brimonidine on Rabbit Trabecular Meshwork Hyaluronidase Activity

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PURPOSE. To study the presence of hyaluronidase activity in the rabbit trabecular meshwork and its regulation by brimonidine.

METHODS. A spectrophotometric assay that consists of the assessment of N-acetylhexosamine groups released from hyaluronic acid was used to examine hyaluronidase activity. Cyclic adenosine monophosphate (cAMP) levels were assessed by radioimmunoassay.

RESULTS. Hyaluronidase activity was detected in the rabbit trabecular meshwork. Its optimal activity was in the acid range of pH 3.8. Brimonidine significantly increased trabecular hyaluronidase-specific activity and decreased cAMP accumulation. Yohimbine significantly inhibited the effect of brimonidine on both hyaluronidase activity and cAMP accumulation.

CONCLUSIONS. The finding of endogenous hyaluronidase activity in rabbit trabecular meshwork supports the hypothesis that this tissue can metabolize its own glycosaminoglycan (GAG) products. The present results suggest, however, that the hypotensive effect of brimonidine could be mediated, at least in part, by its ability to increase GAG catabolism, probably through a cAMP-independent mechanism. (Invest Ophthalmol Vis Sci. 2000;41:2268–2272)

The primary site of aqueous humor outflow resistance resides within the trabecular meshwork and possibly within the deep portion of the corneoscleral meshwork and/or the amorphous juxtanacalicular basement membrane near Schlemm’s canal. The trabecular meshwork is composed of sheets of trabecular beams that contain lamellae made of extracellular matrix materials, which comprise a significant portion of this tissue and probably of the outflow barrier. Among the materials of the trabecular extracellular matrix, glycosaminoglycan (GAG) profile (i.e., hyaluronic acid [HA], keratan sulfate, heparan sulfate, and hybrid dermatan sulfate-chondroitin sulfate) has been identified in rabbits,1 monkeys,2 dogs,9 the evidence suggests that it has little effect on human eyes.10 Further investigations showed that Streptomyces hyaluronidase is considerably more effective than the testicular hyaluronidase-sensitive mucopolysaccharides in the outflow apparatus. Although testicular hyaluronidase has been reported to increase outflow facility in guinea pigs6 and dogs,9 the evidence suggests that it has little effect on human eyes.10 Further investigations showed that Streptomyces hyaluronidase is considerably more effective than the testicular enzyme in the rabbit eye.11 However, no increase in outflow facility was found with acute Streptomyces hyaluronidase treatment in monkeys.12 Intense histochemical staining observed in the various layers of human trabecular meshwork suggests that a substantial amount of HA is present in the outflow pathway.13 and a quantitative analysis has indicated that it is the most abundant GAG of the human trabecular meshwork.2 Although the biosynthesis of acid mucopolysaccharides in trabecular cells has been conclusively demonstrated,14 the mechanism of its degradation remains incompletely understood.

Although the modulation of extracellular matrix materials in the trabecular meshwork by substances such as ascorbic acid15 and glucocorticoids6 has been demonstrated, the effect of medication on the trabecular meshwork biochemistry is an open question; it is not known what if any influence drug therapy may have on the expression of GAGs in this tissue. Brimonidine is a relatively new, highly selective, and potent \( \alpha_2 \)-adrenoreceptor agonist that has been shown to decrease intraocular pressure (IOP), both in the prevention of its elevation after argon laser trabeculoplasty and in long-term control of IOP in patients with glaucoma and ocular hypertension.16–19 A dual effect has been suggested as the mechanism of the hypotensive action of brimonidine: a decrease in aqueous humor production and an increase in uveoscleral outflow.19 Until now, no relationship has been established between this drug and trabecular GAGs. The purpose of the present study was to examine the presence of hyaluronidase.
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METHODS

Reagents and Drugs

Hyaluronic acid, \(\beta\)-dimethyl-aminobenzaldehyde, 3-isobutylmethylxanthine (IBMX), 8-bromoadenosine 3'5'-cyclic monophosphate (8-Br cAMP), and 2'-O-dibutyryladenosine 3'5'-cyclic monophosphate (dibutyryl cAMP) were obtained from Sigma (St. Louis, MO), and yohimbine was obtained from RBI (Natick, MA). Brimonidine tartrate was kindly supplied by Allergan-LOA (Buenos Aires, Argentina).

Animals and Tissues

Male albino rabbits (average weight, 2.5 ± 0.3 kg) were anesthetized with intravenous pentobarbital (40 mg/kg) and killed by an air injection into the marginal vein of the ear. After death, the eyes were quickly enucleated and placed in 0.25 M ice-cold sucrose containing 20 mM Tris-HCl buffer (pH 7.4). To isolate the trabecular meshwork tissues, the sclera was cut off radially from the posterior pole to the equator to remove the vitreous, retina, choroid, and lens. After the tips of the ciliary process had been excised, the iris was carefully cut from the ciliary body. An incision was made at the limbal level in the cornea and the cornea was cut off radially, leaving the scleral spur with the limbal sclera and including the corneoscleral and uveal portions of the trabecular meshwork.

Hyaluronidase Assay System

The trabecular tissues were incubated for 2 hours at 37°C in HEPES-Tris buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with Tris base, in the presence or absence of brimonidine tartrate, yohimbine, or cyclic adenosine monophosphate (cAMP) analogues (8-Br and dibutyryl cAMP). The final concentration of brimonidine was 0.2% wt/vol (4.3 mM). After the medium was removed, the trabecular tissues were minced with scissors, homogenized in 3 ml 0.25 M sucrose-Tris buffer (pH 7.4), and poured onto two layers of fine gauze to filter off the tissue remnants. The filtrate was centrifuged at 1000 g for 10 minutes, and the pellet was discarded. The supernatant was frozen and thawed five times and centrifuged at 12,500g for 20 minutes. The resultant supernatant was dialyzed for 6 hours against 0.1 M acetate buffer (0.9% NaCl, pH 3.8). The nondialyzable material was concentrated in Centricon 10 concentrators (Amicon, Beverly, MA) and used as tissue extract (100–150 \(\mu\)g of protein/tube). The hyaluronidase assay was performed using the modified method of Aronson and Davidson.\(^{20}\) The 0.5-ml reaction system contained 300 \(\mu\)g hyaluronic acid in 250 \(\mu\)l 0.1 M acetate buffer and tissue samples. After 3 to 5 hours at 37°C, the reaction was stopped by raising the reaction pH from 3.8 to 8.9 by adding 10 \(\mu\)l 4 N NaOH and 100 \(\mu\)l 0.8 M potassium tetraborate solution (pH 9.2). The reaction mixture was assayed for \(N\)-acetylglycosamine end groups by the method of Reissig et al.,\(^{21}\) with \(N\)-acetylgalactosamine (5–500 nanomoles) as the standard. After the pH was increased, the mixture was kept in a boiling water bath for 3 minutes, cooled, and treated with 3 ml 1% \(\beta\)-dimethylaminobenzaldehyde reagent in glacial acetic acid containing 1.25% 10 N HCl for 50 minutes at 37°C. Optical density at 585 nm was measured keeping blanks for reagent and substrate. Heat-inactivated tissue extracts were used to assess the nonspecific release of \(N\)-acetylgalactosamine. To determine pH activity profile, the pH of the reaction buffer was adjusted with acetic acid or NaOH. Hyaluronidase activity, expressed in milliunits, was defined as the amount of enzyme that causes the release of 1 nanomole \(N\)-acetylgalactosamine in 1 hour at 37°C. In our experimental conditions enzymatic degradation of hyaluronic acid was linear with time up to 8 hours.

Assay of cAMP Level

Trabecular meshwork tissues were incubated for 30 minutes at 37°C in 3 ml HEPES-Tris buffer containing 0.5 mM IBMX, with or without 0.2% brimonidine, in the presence or absence of yohimbine (final concentration, 0.5 mM). The tissues were homogenized in 1 ml 0.5 mM IBMX and boiled for 2 minutes. The homogenates were cooled and centrifuged at 5000g for 5 minutes at 4°C. The content of cAMP was measured in the supernatants by radioimmunoassay. Aliquots of samples or standards were acetylated with acetic anhydride-triethylamine. The acetylated products were mixed with \([125I]\)-cAMP (15,000–20,000 dpm, specific activity 140 mCi/millimole) and a rabbit antiserum kindly supplied by the National Institute of Diabetes and Digestive and Kidney Disease (1:5000 working solution) and incubated overnight at 4°C. The antigen-antibody complexes were precipitated with ethanol at 4°C using 2% bovine serum albumin as a carrier, centrifuged at 2000g for 20 minutes, and separated by aspirating supernatants. Radioactivity in the pellet was measured in a gamma counter. The range of the standard curve was 10–5000 femtomoles of cAMP. Protein content was determined by the method of Lowry et al.,\(^{22}\) using bovine serum albumin as the standard.

All animal use procedures were in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Hyaluronidase activity was detected in the rabbit trabecular meshwork, using the spectrophotometric assay described. Average specific activity was 100 ± 7 nanomoles/h⋅mg protein. The optimum pH for the trabecular hyaluronidase activity was determined by altering the buffer pH in the enzymatic assay. When hyaluronidase activity was plotted as a function of pH, a major peak of activity was observed at pH 3.8, with a second minor peak in the neutral range (Fig. 1). Brimonidine significantly increased hyaluronidase-specific activity as shown in Figure 2. Yohimbine, although ineffective itself, completely blocked the effect of brimonidine. Because the decrease of cAMP levels is the mechanism classically associated with \(\alpha\)-adrenergic action, the effect of brimonidine on this nucleotide level was assessed. Brimonidine significantly decreased cAMP accumulation (Fig. 3). Yohimbine significantly inhibited the effect of brimonidine on cAMP content. As shown in Figure 2, 0.5 mM 8-Br or dibutyryl cAMP did not modify hyaluronidase activity.

DISCUSSION

Although the presence of hyaluronic acid in the trabecular meshwork is well known, the mechanism of its clearance is not
completely understood. In agreement with a former study by Mayer et al.,23 Laurent and Reed24 reported that HA is not significantly metabolized intracamerally but leaves the anterior chamber gradually by the bulk flow. However, other investigators suggest that various tissues lining the anterior chamber digest the intracameral HA through an intralysosomal hyaluronidase that hydrolyzes the $N$-acetylglucosamine bond. In fact, hyaluronidase activity was measured in human vitreous25 and corneal endothelium26 by an enzyme-linked immunosorbent assay. Hayasaka and Sears, although using a method based on carbocyanine dye binding that has several limitations, reported that lysosomal hyaluronidase activity in the inner layer of rabbit corneoscleral junction containing trabecular meshwork shows the highest specific activity among the corneoscleral tissues.27 Unlike that used in most studies of ocular hyaluronidase activity, the methodology used in the current study allowed us to assess enzymatic activity in absolute units (e.g., the amount of enzyme that causes the release of 1 nanomole $N$-acetylglucosamine in 1 hour at 37°C) and therefore does not rely on exogenous hyaluronidase as a standard for quantification. This methodology has been successfully used for assessment of hyaluronidase activity in several systems, including rat liver,20 goat spermatozoa,28 and human trabecular cell cultures.29 The optimum hyaluronidase activity was in the acid range of pH 3.8 with a second minor activity peak at pH 7. Isolated hyaluronidase from different sources also have an optimum pH in the acid range, indicative of a lysosomal origin. With respect to the eye, both cultured human trabecular meshwork cells,29 human cornea,26 and rabbit cornea and uvea27 have been shown to have acid hyaluronidase activity. The finding of endogenous hyaluronidase activity in rabbit trabecular meshwork further supports the hypothesis that this tissue can metabolize its own GAG products. Because the trabecular meshwork has been reported to have phagocytic activity,30,31 it seems likely that this enzyme may degrade the engulfed endogenous HA. The potential regulation of hyaluronidase by ocular drugs may provide important clues to its function in physiological and pathologic conditions.

Brimonidine is emerging as a first-line therapy for primary open-angle glaucoma with a peak IOP-lowering efficacy comparable with that of timolol, but without an adverse cardiopulmonary side effect, and it offers a more favorable systemic safety profile than that of nonsensitive $\beta$-blockers.52 The present results indicate that 0.2% brimonidine significantly increases hyaluronidase-specific activity in the rabbit trabecu-
lar meshwork. This concentration of brimonidine, although high, is currently used to lower IOP in patients with ocular hypertension and glaucoma.\(^{46}\) The effect of brimonidine was completely blocked by yohimbine, an \(\alpha_2\)-selective antagonist that is ineffective itself. The intracellular events triggered by brimonidine that could account for its effect on trabecular hyaluronidase activity remain to be established. Activation of \(\alpha_2\)-adrenergic receptors inhibits adenylyl cyclase activity and decreases cAMP levels in a number of secretory and absorptive epithelia, including ciliary epithelium of the eye, through a Gi protein–dependent mechanism.\(^{33}\) The presence of functional \(\alpha_2\)-adrenergic receptors has been demonstrated in cultured human trabecular meshwork cells.\(^{34}\) Because brimonidine decreased IBMX-induced cAMP accumulation in rabbit trabecular meshwork, with its effect blocked by yohimbine, it seems likely that this type of adrenergic receptor is also present in rabbit tissue.

It is known that stimulation of adenylate cyclase (e.g., by \(\beta_2\)-adrenergic agonists) leads to an increase in ocular outflow. Therefore, it is expected that inhibition of cAMP synthesis by an \(\alpha_2\)-adrenergic agonist, as is the case with brimonidine, results in decreased outflow. However, it has been demonstrated that brimonidine significantly decreases uveoscleral resistance both in rabbit\(^{35}\) and human,\(^{19}\) contributing to its ocular hypotensive effect. Results in the current study may account for this apparent discrepancy. If a decrease in cAMP levels explains the brimonidine-induced increase in hyaluronidase activity, a reduction of enzymatic activity in the presence of nucleotide analogues could be expected. Thus, because trabecular hyaluronidase activity was unaffected by cAMP analogues, it is possible that brimonidine’s effect could involve a non–cAMP-mediated response. In fact, it has been demonstrated that \(\alpha_2\)-adrenoceptor-mediated contractions of the porcine isolated ear artery is mediated partially by a cAMP-independent mechanism\(^{36}\) and that activation of this adrenergic response inhibits norepinephrine release by a pertussis toxin–insensitive pathway in rat sympathetic neurons.\(^{37}\) The identification of an alternative second messenger for brimonidine action on hyaluronidase activity is under current investigation.

GAGs may reduce the functional diameter of the flow channels through the deep corneoscleral intertrabecular spaces and/or regulate flow through the juxtaocular basement membrane. In addition, it has been shown that mean IOP in the rabbit is proportional to the polymer size of HA.\(^{37}\) Taking into account this evidence, it is tempting to speculate that the effect of brimonidine in increasing outflow could be mediated, at least in part, by its stimulation of hyaluronidase activity, that is by increasing GAG’s clearance. Considering that open-angle glaucoma has been associated with reduced drainage of aqueous humor, knowledge of the mechanism(s) of pharmacologic facilitation of ocular outflow will help the therapeutic challenge faced by ophthalmologists treating glaucoma.

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References