Bremazocine Increases C-Type Natriuretic Peptide Levels in Aqueous Humor and Enhances Outflow Facility

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ABSTRACT

A relatively selective agonist of \( \kappa \) opioid receptors (KOR), bremazocine (BRE), lowers intraocular pressure in rabbits, in part, by increasing natriuretic peptide levels in aqueous humor and by enhancing total outflow facility (TOF). Natriuretic peptide (NP) levels [atrial NP (ANP), brain NP (BNP), and C-type NP (CNP)] were measured in aqueous humor of rabbits either by radioimmunoassay or enzyme immunoassay. TOF was determined in rabbits by two-level constant pressure perfusion of the anterior chamber. Experimental regimens included topical treatment with BRE in the presence or absence of KOR antagonist (norbinaltorphimine), protein kinase C inhibitor (chelerythrine), and natriuretic peptide receptor antagonist (isatin). The rank order of basal NP levels in aqueous humor of rabbits was BNP \( \gg \) CNP \( > \) ANP. Topical administration of BRE (1–100 \( \mu \)g) caused dose-related elevations of CNP levels in aqueous humor that were inhibited by topical pretreatment with either norbinaltorphimine (100 \( \mu \)g, bilaterally) or chelerythrine (10 \( \mu \)g, bilaterally). Topically administered BRE (100 \( \mu \)g) also elevated levels of ANP and BNP in aqueous humor and evoked an 80% increase in TOF. The increase in TOF was antagonized by topical pretreatment with either norbinaltorphimine (100 \( \mu \)g, bilaterally) or isatin (100 \( \mu \)g, bilaterally). Bremazocine induced an increase in NP (ANP, BNP, and CNP) levels and TOF in rabbits by activating KOR. The increase in CNP levels elicited by BRE was inhibited by norbinaltorphimine and chelerythrine; therefore, this event is most likely mediated by a KOR-linked activation of protein kinase C. These data provide evidence that the increase in TOF elicited by BRE was mediated by a KOR-activated paracrine effect of NPs on tissues within ocular outflow tract(s).

Neuroendocrine regulation of fluid homeostasis throughout the body depends, in part, on the activity of the natriuretic peptide system. Of the three natriuretic peptides [i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)], the sequences of ANP and CNP are more highly conserved among the species, whereas the structure of BNP varies greatly. It has been proposed that natriuretic peptides generated within the eye contribute to the regulation of aqueous humor dynamics; however, the understanding of how this ocular neuroendocrine system is modulated is very limited.

Recently, molecular evidence has been presented for neuroendocrine functions in the ciliary epithelium (Coca-Prados et al., 1999; Ortego and Coca-Prados, 1999). Moreover, it has been postulated that natriuretic peptides released by drugs from ciliary epithelial cells could then act in an autocrine and/or paracrine manner to change intraocular pressure (IOP). Recent reports have shown that ocular hypotensive drugs such as \( \kappa \) opioid receptor (KOR) agonists (bremazocine) (Russell et al., 2001; Russell and Potter, 2002) and imidazoline-1/2 agonists (Ogidigben et al., 1999, 2002) elevate ANP levels in aqueous humor of rabbits.

The current study was conducted to: 1) determine the relative basal levels of natriuretic peptides in the aqueous humor of rabbits, 2) explore the possible involvement of activation of KOR and protein kinase C (PKC) in bremazocine (BRE)-induced elevation of CNP levels, 3) demonstrate the effects of BRE on outflow facility in rabbits, and 4) determine whether the observed changes in outflow facility can be suppressed by antagonizing activity at \( \kappa \) opioid and natriuretic peptide receptors. In this regard, BRE was tested topically, in the absence and presence of antagonists, for the ability to elevate CNP levels in aqueous humor and to enhance outflow facility. Subsequently, topical pretreatment with antagonists (isatin, norbinaltorphimine) was used to confirm the roles of natriuretic peptide receptors and KOR, respectively, in
bremazocine-induced increases in outflow facility. Topical pretreatment with chelerythrine was utilized to determine whether bremazocine-induced release of CNP involved activation of PKC.

Materials and Methods

Animals. New Zealand White (NZW) male and female rabbits (2–4 kg b.wt.) were used for all experimental procedures. The rabbits were purchased from Myrtle Rabbitry, Thompson Station, TN. Animals were maintained in a temperature-regulated room with food and water ad libitum. All rabbits were housed individually, and their care and treatment were conducted in accordance with guidelines adopted and promulgated by the National Institutes of Health and recommended by the Association for Research in Vision and Ophthalmology on the use of animals for research. The Institutional Animal Care and Use Committee of Morehouse School of Medicine and the Medical University of South Carolina approved all protocols involving animals.

Measurement of Natriuretic Peptide Levels in the Aqueous Humor. Rabbit eyes were treated topically and bilaterally with BRE (1, 10, and/or 100 μg/25 μl), a κ opioid receptor agonist. At 0.5 h after treatment, the rabbits were sacrificed by an overdose of Beuthansia-D (pentobarbital sodium) and the aqueous humor removed by paracentesis. The aqueous humor was placed immediately on ice in microfuge tubes containing a protease inhibitor mix (Sigma-Aldrich, St. Louis, MO; 10 μl/200 μl of aqueous humor). In control (vehicle-treated) animals, the vehicle (saline) was applied topically to both eyes, and the sampling schedule was the same as those of the treatment group. The topical pretreatment with antagonists norbinaltorphimine (norbNBI, 100 μg/25 μl; Torcia Cookson Inc., Ballwin, MO) or chelerythrine chloride (10 μg/25 μl; Sigma-Aldrich), a PKC inhibitor, preceded the challenge with BRE by 0.5 h. The topical doses of antagonists were determined from previous experimentation in rabbits (Russell et al., 2000) or were extrapolated from previously published in vivo doses in rabbits (Sandhu et al., 1997) and monkeys (Tian et al., 2000).

Both the enzyme immunoassay (EIA) and radioimmunoassay (RIA) analyses for natriuretic peptide levels were performed according to the manufacturer’s instructions. The level of BNP in aqueous humor was determined using an EIA kit purchased from Peninsula Laboratories, Belmont, CA. Before the assay, BNP was extracted from the aqueous humor using Varion Bond Elut C8 columns. Before sample application, the columns were prepared by washing with several volumes of methanol and water. Aqueous humor samples (200 μl) were then applied to the columns followed by rinsing sequentially with saline (2 ml), water (4 ml), and methanol (1 ml). Samples were eluted with 80% acetonitrile in 0.4% trifluoroacetic acid (1 ml). The BNP isolates were then evaporated to dryness in a SpeedVac drier. The pellet obtained from the BNP extraction was reconstituted in 200 μl of EIA buffer.

Because both direct and extracted assays indicated similar changes in natriuretic peptide levels (Guillaume et al., 1994), the amount of CNP or ANP in the aqueous humor samples was quantified using a RIA kit purchased from Peninsula Laboratories, Inc. The details of the RIA have been described previously (Russell et al., 2001). Levels of natriuretic peptides were determined from the individual standard curves and expressed as picograms per milliliter of aqueous humor. All standard and sample assays were performed in duplicate.

Measurement of Total Outflow Facility. Total outflow facility was determined by two-level constant pressure perfusion of the anterior chamber (3 and 13 mm Hg above spontaneous IOP) with Barany’s (1964) mock aqueous humor (8 g/liter NaCl, 0.35 g/liter KCl, 0.17 g/liter CaCl2, 64 mg/liter MgCl2, 69 mg/liter Na2PO4, 13.7 mg/liter NaH2PO4, and 1 g/liter glucose) as described previously (Crosson, 2001). Initially, rabbits were treated topically with BRE (100 μg) or vehicle alone to determine the effect of the agonist on total outflow facility. To determine the involvement of KOR and natriuretic peptide receptors, bilateral pretreatment with norbinaltorphimine (100 μg/eye) or isatin (100 μg/eye) topically was performed 30 min before the challenge with BRE. Rabbits were then anesthetized with 33 mg/kg ketamine and 6 mg/kg xylazine intramuscularly, and the cornea was anesthetized by the topical application of 50 μl of 0.5% proparacaine. To minimize breakdown of the blood-aqueous barrier, rabbits were pretreated with indomethacin (50 μl, 0.2% topically) 1 h before cannulation of the anterior chamber with a 25-gauge 3/8-inch needle. After obtaining baseline data, outflow facility was then measured multiple times between 1 and 2 h for the contralateral (vehicle-treated) eye and 2 to 3 h for the ipsilateral (BRE-treated) eye. During this period, four to five facility measurements were obtained and averaged to give the final facility value for each eye. All facility measurements were corrected for internal resistance of the perfusion apparatus.

Statistical Analysis. Each experiment involving natriuretic peptide or outflow facility determinations was repeated at least four times. The statistical analysis of the experimental data utilized either the two-way analysis of variance (ANOVA) or the Student’s t test. Graphed values represent the mean ± S.E. The minimum level of probability accepted as significant was P < 0.05.

Results

Basal and BRE-Induced Changes in Natriuretic Peptide Levels. This portion of the study was designed to: 1) compare basal levels of natriuretic peptides in the aqueous humor of rabbits, 2) determine whether topically administered BRE elevates levels of BNP and CNP in aqueous humor, and 3) ascertain the possible involvement of KORs and PKC in BRE-induced elevation of CNP levels.

As shown in Fig. 1, the rank order of the basal natriuretic peptide levels in the aqueous humor of NZW rabbits was BNP ≫ CNP > ANP. The basal level of BNP (424 pg/ml) was approximately 14- to 56-fold higher than the levels of CNP and ANP, respectively. The basal level of CNP was approximately four times greater than that of ANP (29.5 versus 7.5 pg/ml).

In the previous experimentation with rabbits, BRE was shown to elevate ANP levels in a dose-related manner from a basal level of approximately 7 pg/ml (basal) to 15 (10 μg) and 28 pg/ml (100 μg) (Russell et al., 2001). In the current set of experiments, topical application of BRE (1–100 μg) increased CNP levels in a dose-related manner (Fig. 2). At a concentra-

![Fig. 1. Basal levels of natriuretic peptides (ANP, CNP, and BNP) in the aqueous humor of NZW rabbits. Plotted values represent the mean (picograms per milliliter) ± S.E.](image-url)
tration of 100 μg, the BRE-induced change in CNP levels (61.5 pg/ml) was approximately double the control level (29.5 pg/ml). In other experiments, BRE (100 μg) caused an increase of approximately 4-fold (67.5 ± 6.3 to 286.9 ± 17.8 pg/ml, n = 4 and 8, respectively) in BNP levels in aqueous humor. Thus, at the comparable dose of 100 μg, the ascending order of increase in natriuretic peptide levels evoked by BRE was ANP < CNP < BNP.

To confirm that the BRE-induced elevation of CNP levels was the result of KOR activation, rabbits were pretreated topically with norbinaltorphimine (100 μg, bilaterally) before a challenge with BRE (100 μg). Norbinaltorphimine had no effect on basal levels of CNP (data not shown) but as shown in Fig. 3, it antagonized the ability of BRE to elevate CNP levels in aqueous humor.

Because ANP expression and/or release has been associated with PKC activation (Church et al., 1994; Tokola et al., 1994; Lenz et al., 1999) in cardiac and renal tissues, it was deemed appropriate to determine whether a PKC inhibitor, such as chelerythrine, could suppress BRE-induced elevation of CNP levels in the eye. Data shown in Fig. 4 demonstrate that pretreatment with chelerythrine (10 μg, bilaterally) reduced significantly the ability of BRE to elevate CNP levels in aqueous humor. Treatment with chelerythrine alone had no effect on basal CNP levels in the aqueous humor of NZW rabbits (data not shown).

**Bremazocine-Evoked Increase in Total Outflow Facility: Involvement of KOR and Natriuretic Peptides.** The ability of BRE to enhance outflow facility in rabbits is shown in Fig. 5. The basal outflow facility in the vehicle-treated eyes ranged between 0.162 and 0.211 μl/min/mm Hg. Although BRE was applied unilaterally, significant changes in outflow facility were observed in both eyes. In the contralateral and ipsilateral eyes, total outflow facility increased 0.108 and 0.169 μl/min/mm Hg, respectively.

To validate that the BRE-induced increase in total outflow facility was mediated by KOR, rabbits were pretreated bilaterally with a relatively selective KOR antagonist, norbinaltorphimine, 30 min before the challenge with BRE. As observed in Fig. 6, norbinaltorphimine (100 μg/eye) elicited no appreciable change in total outflow facility when compared with saline treatment; however, the response to BRE (100 μg) was significantly suppressed by pretreatment with norbinaltorphimine.

Natriuretic peptides increase outflow facility in the rabbit eye when injected intravitreally (Takashima et al., 1996, 1998). According to Takashima and colleagues (1996) and Fernandez-Durango and coworkers (1999), the intravitreal injection of CNP lowers intraocular pressure more effectively than either ANP or BNP. As shown in this and previous studies (Russell et al., 2001), BRE raises the levels of CNP, BNP, and ANP in the aqueous humor of rabbits. Hence, it was hypothesized that the BRE-induced increase in outflow facility could be due, in part, to a paracrine consequence of natriuretic peptide release. Figure 7 illustrates that when...
rabbit eyes were pretreated with the natriuretic peptide antagonist, isatin (100 µg, bilaterally), and then challenged with BRE (100 µg) the outflow facilitating effect of BRE was abrogated; however, isatin alone had no appreciable effect on total outflow facility.

Discussion

Although it has been known for some time that the genes for natriuretic peptides are expressed at extracardiac sites, Fernandez-Durango and coworkers (1995) were among the first to demonstrate that the messenger RNAs for natriuretic peptides and their receptors are expressed in the eyes of rabbits. Previously, it had been demonstrated that exogenously administered natriuretic peptides lower IOP in rabbits (Stone and Glembotski, 1986; Sugrue and Viader, 1986) presumably by enhancing outflow facility (Takashima et al., 1996, 1998). As in other tissues, it seems plausible that the expression and secretion of natriuretic peptides in the eye can be altered both in vivo and in vitro by a variety of humoral, neural, and mechanical stimuli (Ruskoaho, 1992). Fernandez-Durango and colleagues (1990, 1991) demonstrated that ANP levels increased significantly when IOP was elevated in rabbits. Moreover, preliminary studies in humans have shown ocular hypotensive effects of administered ANP (Diestelhorst and Kriegelstein, 1989) and of candostatril, a neutral endopeptidase inhibitor that inhibits the degradation of ANP (Wolfensberger et al., 1994).

The hypothesis that the effect of ocular hypotensive drugs could be mediated, in part, by the elevation of natriuretic peptide activity in aqueous humor was suggested by the work of Fernandez-Durango and coworkers (1995) and Ortego and Coca-Prados (1999). It is of interest that there are multiple steps in the natriuretic peptide cascade whereby drugs can alter the steady-state activity of the peptides including release and clearance. Thus, the ability of drugs to exert a positive influence on the natriuretic peptide system in ciliary nonpigmented epithelial cells could represent a therapeutic approach to regulating aqueous humor dynamics at a variety of sites in the anterior segment. One potential target for a drug-induced elevation of natriuretic peptide activity is the trabecular meshwork cell.

This study confirms that basal levels of ANP, BNP, and CNP can be detected in the aqueous humor of NZW rabbits with normal IOPs. As demonstrated in this study, the natriuretic peptide demonstrating the highest level in aqueous humor of rabbits is BNP. Interestingly, the predominant natriuretic peptide produced by nonpigmented ciliary epithelial cells (Ortego and Coca-Prados, 1999), and found in the aqueous humor of the human eye, is also BNP (Salzmann et al., 1998). Because BNP is cleared more slowly than ANP, this might account, in part, for the higher concentrations of BNP in aqueous humor of rabbits and humans (DeBold and Bruneau, 2000). In this regard at equivalent concentrations (10 µg, intracameral), BNP produced a more prolonged lowering of IOP in rabbits than either ANP or CNP; however, C-ANP, which has high affinity for the clearance receptor (NPR-C), had a longer duration of effect than BNP (Fernandez-Durango et al., 1999). Nevertheless, two studies have shown that the intravitreal injection of CNP lowers intraocular pressure in rabbits more efficaciously than either ANP or BNP (Takashima et al., 1996; Fernandez-Durango et al., 1999).

Ortego and Coca-Prados (1999) identified the molecular machinery for the generation of ANP and BNP in transformed human nonpigmented epithelial cells but were unable to identify components related to CNP. In contrast, significant basal levels of CNP were detected in the aqueous humor of rabbits.
humor of rabbits. Therefore, it was of interest to determine whether BRE caused dose-related elevation of CNP levels in the aqueous humor of rabbits. In this study, BRE (100 μg) increased CNP to a level (60 pg/ml) that was greater than the maximal level for ANP (Russell et al., 2001); however, the basal level of ANP (7.5 pg/ml) was considerably lower than CNP (29.5 pg/ml) such that the increase elicited by BRE was greater for ANP (approximately 3- to 4-fold). Nevertheless, the greatest absolute increase in natriuretic peptide levels promoted by BRE involved BNP (approximately 180 pg/ml).

Because BRE’s selectivity for KORs is dependent upon dose, it was of interest to determine whether the elevation of CNP levels could be inhibited by norbinaltorphimine, an antagonist of KORs. Topical pretreatment with the antagonist abolished the BRE-induced increase of CNP levels. These results support the idea that the CNP-elevating effect of BRE was the product of its interaction with KORs.

In cardiac tissues, PKC activation increases ANP release (Ruskoaho, 1992). Previous results from this laboratory have shown that KOR agonists can activate PKC-related mechanisms in the iris-ciliary body of the rabbit (Dortch-Carnes and Potter, 2002). To determine whether an inhibitor of PKC could attenuate BRE-induced elevation of CNP levels in aqueous humor, rabbits were pretreated with chelerythrine. As demonstrated in this study, pretreatment with chelerythrine a PKC inhibitor antagonized the increase in CNP levels as effectively as norbinaltorphimine. These data support the suggestion that activation of KOR by BRE in the anterior segment facilitates PKC-dependent release of natriuretic peptides.

The next phase of the study was to determine whether topical administration of BRE could elicit an increase in outflow facility. The rationale for these experiments was based on the possibility that BRE-induced release of natriuretic peptides could exert an effect at the level of the trabecular meshwork cells in the conventional outflow tract. As shown by experiments in this study, BRE can produce a significant increase in total outflow facility in the rabbit eye. Several questions arose from this observation. 1) Is the effect of BRE on outflow facility mediated by KORs? 2) Do natriuretic peptides contribute to the effect of BRE on outflow facility? To answer these questions, experiments with norbinaltorphimine (a relatively selective antagonist of KORs) and isatin (an antagonist of natriuretic peptide receptors) were initiated; eyes were pretreated topically with these inhibitors before being challenged with BRE.

Although treatment with norbinaltorphimine alone had no effect on total outflow facility, it was able to antagonize the BRE-induced increase very effectively. These data demonstrated the involvement of KORs in the BRE-evoked increase in outflow facility but did not address whether the response involved natriuretic peptides. As alluded to previously, intra-vitreal injection of natriuretic peptides can increase outflow facility in rabbit eyes (Takashima et al., 1996, 1998).

Isatin is an endogenously occurring compound that can inhibit natriuretic peptide receptor activation (Glover et al., 1995) possibly by allosteric means (Medvedev et al., 1998, 1999). Although isatin alone exerted no appreciable effect on outflow facility in the rabbit, it antagonized the ability of BRE to increase outflow facility. These data suggest that BRE elevates natriuretic peptide levels in aqueous humor and that these peptides can act in a paracrine fashion to increase total outflow facility. However, these findings do not identify whether the predominant effect is mediated by effects on the conventional outflow tract (trabecular meshwork) or by the uveoscleral outflow pathway (ciliary muscle).

In summary, these results confirm that three natriuretic peptides (ANP, BNP, and CNP) are present in the aqueous humor of rabbits. In addition to releasing ANP, BRE (a KOR agonist) can evoke dose-related release of CNP. BRE-induced release of CNP can be suppressed by norbinaltorphimine (a relatively selective KOR antagonist) and chelerythrine (a relatively selective PKC inhibitor). Topically administered, BRE caused an increase in total outflow facility that was antagonized by pretreatment with either norbinaltorphimine or isatin (an inhibitor of natriuretic peptide receptor activation).

In conclusion, these findings provide convincing evidence that activation of KOR in the anterior segment can elevate natriuretic peptide levels in aqueous humor of rabbits and that the enhanced egress of aqueous humor caused by BRE in rabbits is mediated, in part, by a paracrine effect of natriuretic peptides on outflow pathways.

Acknowledgments

We recognize with gratitude the skilled technical assistance of Dr. Phillip Yates in conducting the outflow facility measurements and Dr. Luanna Bartholomew in preparing the submitted manuscript. We are grateful to Drs. Craig E. Crosson and Miguel Coca-Prados for discussions about the experimental data generated by the study. The authors have no financial or proprietary interest in any product mentioned herein.

References


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