Natriuretic peptide system in the human retina

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Purpose: The natriuretic peptide (NP) family includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Natriuretic peptides are known to inhibit vascular cell growth and regulate vessel tone. There is also much evidence that they modulate vascular permeability and angiogenesis, as well as regulating aqueous humor production in the eye. All these data indicate that the natriuretic peptide system might be involved in the development of diabetic retinopathy and glaucoma. Given the expression pattern of natriuretic peptides (NPs) and their receptors, natriuretic peptide receptor A (NPRA), natriuretic peptide receptor B (NPRB) and natriuretic peptide receptor C (NPRC) in the human retina has not yet been established, the present study was designed to determine ANP, BNP and CNP gene expression and localize the mature peptides in this tissue. The expression pattern of the genes encoding the different NP receptor subtypes was also examined.

Methods: Eyes (n=10) from human donors with no history of eye disease were fixed and processed for routine paraffin embedding. The cellular location of the NPs was established by immunohistochemistry. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate the expression of NP and NP receptor genes in neural retinas obtained from the contralateral eyes.

Results: Immunohistochemistry revealed the presence of NPs in the neural retina and retinal pigment epithelium. Positive NP immunostaining was observed within the astrocytes and in their processes enveloping vessels. In the anterior portion of the optic nerve, NPs were intensely labeled in neural bundles. We were able to detect NP gene expression in the human retina. The levels of NP receptor-encoding transcripts detected indicated no significant differential expression of genes coding for the different receptor subtypes.

Conclusions: Our finding that NP receptor transcripts are expressed along with ANP, BNP, and CNP mRNA in the human retina provides evidence for a local system in this tissue. The expression of NPs in neural retinal, glial, and vascular elements of the normal adult retina suggests a role for these peptides in maintaining both the neural and vascular integrity of the mature retina.

The natriuretic peptide (NP) family is formed by atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). NPs occur in many other tissues, such as brain, lung, and organs of the gastrointestinal and immune systems [12,13]. In the eye, immunoreactive ANP has been demonstrated in rat and rabbit retinas and in rabbit choroid tissue by radioimmunoassay (RIA), [14,15]. Palm et al. [16] immunohistochemically detected ANP in the outer and inner plexiform layers of the retina in rats and rabbits. ANP binding sites were found in rat [17,18] and rabbit [17] retinas by binding assay and autoradiography. The molecular cloning of NPRA was reported in human and rat retina [19,20]. Moreover, Duda et al. reported the presence of NPRB by molecular cloning in human retina [21]. NPRA mRNA has been detected in rat retina by RT-PCR [22,23]. In addition, Fernández-Durango et al. [24] demonstrated the presence of mRNA transcripts encoding NPRA, NPRB and NPRC in the retina, choroid and ciliary process of the rat and rabbit eye.
Several reports have demonstrated that ANP is able to regulate aqueous humor production in the eye [17,25-27]. Fernandez-Durango et al. [28] reported that NPs decreased intraocular pressure and stimulated guanylate cyclase activity in the rabbit ciliary body, and that the NPRC receptor modulates the NP concentration of the aqueous humor. Moreover, ANP acts as an anti-permeability factor by inhibiting the signaling functions of vascular permeability factors, and preserving endothelial cell tight junction functional morphology [29]. NPs have been shown to inhibit vascular cell growth and regulate vessel tone [9], and may play an important modulatory role in angiogenesis [11]. Collectively, these data suggest that the NP system might be involved in the development of glaucoma and diabetic retinopathy.

The expression of NP mRNA and NP receptors in the human retina is yet unknown and the location of ANP, BNP, and CNP immunoreactivities of retinal tissue have not yet been well defined. Thus, the aims of the present study were to: localize ANP, BNP, and CNP by immunohistochemistry, evaluate the expression of transcripts coding for ANP, BNP, and CNP and determine the expression profile of the different NP receptor subtypes (NPRA, NPRB, and NPRC) by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

**METHODS**

**Tissue specimens:** Human donor eyes were provided by the Tissue Bank at the Hospital Clinico San Carlos, Madrid, Spain. The number of donors was 10 (7 male and 3 female). Mean donor age was 43 years (range, 13-76). The mean time interval between death and enucleation of the eyes was 5 h (range, 3-7), and between enucleation and fixation was 2 h (range 1-4).

The donors were subjects without diabetes or an ocular disease, as determined by their clinical record. All research procedures involving humans were conducted in accordance with institutional guidelines on the Declaration of Helsinki. After fixation in 10% formalin, the eyes were processed for standard paraffin embedding. The tissue was sectioned at 4 µm and dried on snowcoat-X-tra slides (Surgipath, Winnipeg, Manitoba, Canada) for immunohistochemistry. Contralateral eyes were used for real-time quantitative RT-PCR. Initially, the anterior segment of each donor eye was removed and the posterior poles examined for evidence of an ocular pathology with the aid of the binocular stereomicroscope. Next, the neural retina was carefully peeled away from the RPE-choroid-sclera using a brush. Then, the RPE with adherent choroid was peeled away from the remaining choroid and sclera. Tissues were immediately frozen in liquid nitrogen and stored at -70 °C until use.

**Immunohistochemical detection of ANP, BNP, CNP, and GFAP:** For light microscopy immunohistochemistry we used a mouse monoclonal antibody against human ANP (Cymbus Biotechnology LTD, Hampshire, UK) with specificity for the whole molecule and residues 4-28; a rabbit polyclonal antiserum against human BNP-32 (Peninsula Laboratories, Belmont, CA); a rabbit polyclonal antiserum against human CNP (amino acid sequence from the complete CNP (1-53); Peninsula Laboratories); and a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, clone 6F2; DAKO Corp. Carpenteria, CA). Commercially available polyclonal anti-CNP (antibody recognizes amino acids 32-53 of the complete CNP sequence of 53 amino acids) serum shows 100% cross-reactivity with CNP (amino acids 32-52). Both anti-BNP and anti-CNP antisera show no cross-reactivity to either ANP and BNP or ANP and CNP, respectively. Deparaffinated and hydrated sections were incubated in blocking solution TBT (Tris Base Saline (TBS), 0.5 M pH 7.4 containing 3% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100) for 30 min at room temperature to reduce non-specific binding. Only the anti-ANP antibody was subjected to a preliminary heat-induced antigen retrieval step. This involved pressure cooker heating in a 0.1 M sodium citrate solution prior to incubation with the primary antibodies. Sections were incubated overnight at 4 °C in a humidified chamber with the anti-ANP monoclonal antibody at a 1:50 dilution, the anti-BNP and anti-CNP polyclonal antibodies at a 1:1000 dilution, and an anti-human glial fibrillary acidic protein (GFAP) mouse monoclonal antibody at a 1:50 dilution. The slides were washed for 5 min in TBS. Immunodetection was performed using biotinylated antimouse and anti-rabbit immunoglobulins followed by alkaline phosphatase-conjugated streptavidin and a fuchsin chromogen (LSAB2 kit, DAKO Corp.) for red staining. The sections were lightly counterstained with Mayer’s hematoxylin. Final mounting was done in the water-soluble medium Glicergel (DAKO Corp.).

Negative control slides were incubated either with primary antibody preabsorbed with 10 nM ANP, BNP, CNP, or with normal rabbit serum instead of the primary antibody. Specimens of atrium, ventricle, and renal medulla were used to assess the immunohistochemical detection of ANP, BNP, and CNP, respectively.

**Image analysis:** Images were captured using Leica Qwin image processing and analysis software (Leica microscopy system, Heerbrugg, Switzerland) on a personal computer linked to a high resolution video camera (Leica DC100) mounted on a microscope (Zeiss, Oberkochen, Germany). The results provided were derived from the examination of at least three sections per eye.

**Real-time quantitative RT-PCR:** Total RNA was extracted from retinal tissues using the RNaseasy Mini kit (Qiagen, Santa Clara, CA) according to the manufacturer’s recommendations.

<table>
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<th>Primer Name</th>
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<th>Reverse Primer (5'-&gt;3')</th>
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</table>

GenBank accession number, sequence of PCR primer pairs, anticipated size of the amplified product for the natriuretic peptides, their receptors, and β-actin.
The RNA concentration was spectrophotometrically determined. Following DNase treatment, first-strand cDNA was synthesized using 2.5 μM random hexamers and 1.25 U/μl multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

Real-time quantitative RT-PCR analysis was performed using an automated sequence detection instrument (Prism 7700 Sequence Detector; Applied Biosystems) for the real-time monitoring of nucleic acid green dye fluorescence (SYBR Green I; Applied Biosystems Inc.).

A series of PCRs was performed on retinal cDNAs using primer pairs for ANP, BNP, and CNP. The three different natriuretic peptide receptors: NPRA, NPRB, and NPRC were detected by quantitative real-time RT-PCR. Atrial, ventricular and renal medullary tissues were used as positive controls for ANP, BNP, and CNP, respectively. Ventricular tissue was used as the positive control for NPRA, NPRB, and NPRC.

Primer pairs were designed with the assistance of Prism 7700 sequence detection software (Primer Express, Applied Biosystems). Details of the primers and the GenBank accession numbers are given in Table 1. Specificity was checked in a BLAST search (BLASTNhr) on 21 February 2002, which compared against all databases available. The primers designed shared 100% homology with the target sequences but no significant homology with any other sequences.

PCR was performed using a kit (Applied Biosystems) as previously described [30]. Briefly, the PCR mixture contained 12.5 μl 2X SYBR Green PCR Master Mix, 5 μl (10 ng) of RT product, and 300 nM of the primers in a total volume of 25 μl. PCR amplification was performed according to the temperature profile: 2 min at 50 °C for AmpErase followed by 10 min at 95 °C to inactivate the AmpErase and activate the Ampli Taq Gold DNA polymerase. The cycling conditions were: 15 s melting step at 95 °C followed by 40 cycles of annealing-extension at 60 °C for 1 min. All reactions were performed in duplicate. For each primer pair, non-template controls were included to check for significant levels of contaminants and the formation of primer-dimers; these interfere with quantification, when SYBR Green is used as the fluorescent dye. Agarose gel electrophoretic analysis was used to check the amplified products corresponded to the size predicted for cDNA fragments of the natriuretic peptides (ANP, BNP, and CNP) and their receptors (NPRA, NPRB, and NPRC).

Data analysis: Data were analyzed using the relative standard curve method described in the PE Applied Biosystems User Bulletin number 2 (ABI part number 04303859B). Standard curves were generated in parallel for all three NPRs and β-actin using cDNAs synthesized from serial 1:10 dilutions of an RNA sample, prepared by pooling a fraction of the RNAs of all the individual samples included in this study. For each sample, the amounts of NPRA, NPRB, NPRC, and β-actin were determined from the standard curves. The resulting NPRA, NPRB, and NPRC amounts were divided by the β-actin amount to obtain a normalized value. The results of each PCR experiment were confirmed in a minimum of three consecutive experiments. To validate RT-PCR, standard curves with correlation coefficients more than 0.98 and slope values between -3.1 and -3.4 were required, indicating a near 100% reaction efficiency (E). The Kolmogorov-Smirnov and Shapiro-Wilks tests were used to confirm the assumption of normality of the data. Nevertheless, because the sample populations examined were small, multiple pairwise comparisons were made with the non-parametric Friedman test. Linear correlation between the NPRs or β-actin gene expression and the time to enucleation, post-mortem interval, and donor age was assessed by Spearman correlation. SPSS version 11.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis.

RESULTS

Localization of ANP, BNP, CNP, and GFAP immunoreactivities in the human retina: Specific and intense immunolabelling for ANP (Figure 1A), BNP (Figure 1B), and CNP (Figure 1C) was observed in the nerve fiber layer, the ganglion cell layer, and inner and outer plexiform layers of the human retina. Labeling for the three peptides was also detected in the inner nuclear layer and in scattered cells of the outer nuclear layer. Labeling was observed in the cytoplasm of ganglion cells. Rod and cone inner and outer segments were not labeled. The cells of the retinal pigment epithelium showed strong ANP, BNP, and CNP staining (Figure 1D-F, respectively). Cells of the RPE near the ora serrata showed most intense ANP immunolabeling. In the peripheral retina, ANP, BNP, and CNP labeling was more intense than in the central retina.

GFAP positive astrocytes were found to be mostly located in the nerve fiber and ganglion layers and could be seen to make contact with the adventitia of large vessels through their processes (Figure 1I). In an adjacent section, ANP immunoreactivity was also detected in the cell body of the astrocytes and in their processes enveloping the vessel (Figure 1H). BNP and CNP labeling were similarly detected within the astrocytes and their processes surrounding the vessels.

In the anterior portion of the optic nerve, neural bundles showed intense ANP, BNP, and CNP labeling (Figure 1L-N, respectively). Positive immunostaining for ANP, BNP, and CNP was abolished when the sections were incubated with antigen-absorbed antibodies or with normal rabbit serum instead of the primary antibody (Figure 1J-K).

ANP, BNP, and CNP mRNA expression in the human retina: Figure 2 shows the amplification products obtained from retinal cDNA using pairs of primers specific for ANP, BNP, and CNP. In each case, single bands of the predicted molecular size were obtained: 228 bp, 73 bp, and 61 bp, respectively. When the PCR products were purified and their sequences determined, 100% homology with their respective cDNA sequences was confirmed.

Quantification of NPRA, NPRB, and NPRC mRNA in the human retina: The real-time detection of dsDNA enabled us to construct a dissociation curve at the end of the PCR run by ramping the temperature of the sample from 60 °C to 95 °C while continuously collecting fluorescence data. The curves of the melting profiles for the natriuretic peptide receptors and


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Figure 1. Localization of ANP, BNP, and CNP immunoreactivities. ANP, BNP, and CNP immunoreactivities were examined in human retina and the anterior portion of the optic nerve. Immunostaining appears in red. Positive ANP, BNP, and CNP (A-C, respectively, hematoxylin, x160) labeling can be seen in the inner and outer plexiform and nuclear layers of the retina. The cytoplasm of ganglion cells was also positive for the ANP, BNP, and CNP antibodies (arrows in A-C, respectively; BNP labeling is shown at higher magnification in G). RPE cells also showed intense labeling for ANP (D), BNP (E), and CNP (F; vertical arrows, hematoxylin, x400). ANP and GFAP immunoreactivity could be observed on astrocytes in adjacent sections (arrows, H, I, respectively, hematoxylin, x400) of the retina. Intense ANP, BNP, and CNP labeling (L-N, respectively, hematoxylin, x63) could also be seen in neural bundles of the anterior portion of optic nerves. Negative controls for the immunohistochemical detection of ANP, BNP, and CNP in the retina (J, hematoxylin, x160) and anterior portion of the optic nerve (K, hematoxylin, x63) were free of labeling. The meanings of the abbreviations used in this figure are: RPE (retinal pigment epithelium), OS (photoreceptor outer segments), IS (photoreceptor inner segments), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cells layer), NFL (nerve fiber layer).
the housekeeping gene did not reveal an accumulation of primer dimers (data not shown). Figure 3 shows electrophoresis of the real-time PCR products of NPRA, NPRB, and NPRC. The bands obtained were of the expected size: 98 bp, 75 bp, and 79 bp, respectively.

Our results show that all the NP receptors are expressed in human retinal tissue. The NPR expression profile normalized for β-actin was fairly uniform in the retina specimens. For the final analysis, total NPR gene expression was calculated and set at 100%. Subsequently, the relative contributions of each receptor subtype as a fraction of this total were determined by calculating the percentage corresponding to the mean of the retina specimens. Table 2 and Figure 4 provide the results of this analysis. NPRA, NPRB, and NPRC mRNA expression levels relative to β-actin took the order: NPRA, NPRB, NPRC (from highest to lowest). However, these mRNA levels were not significantly different (means±standard deviations: NPRA 34.60 ±9.50, NPRB 33.52 ±8.40, and NPRC 31.80 ±8.93; Friedman test, p>0.05).

In the small population we examined, the time to enucleation and the postmortem interval showed no correlation (Spearman correlation, p>0.05) with β-actin, NPRA, NPRB, and NPRC gene expression. Neither was any correlation (Spearman correlation, p>0.05) with age noted in the range examined (13-76 years).

DISCUSSION

The present study is the first demonstration by real time quantitative RT-PCR of the expression of the NP system mRNA (ANP, BNP, CNP and their receptors, NPRA, NPRB, and NPRC) in human retinal tissue. Localization of ANP, BNP, and CNP immunoreactivities in the different layers of the human retina and optic nerve were determined by immunohistochemistry.

We detected ANP, BNP, and CNP gene expression in human retinas. In previous molecular studies [15,24], we were able to identify BNP and CNP mRNA but not ANP mRNA in the rat retina by RT-PCR. However, we recently detected ANP in the rat retina by real time quantitative RT-PCR (data not yet published). These differences in our findings most likely reflect the different methodologies used. Indeed, the present study differs from the report by Gaspar et al. [15] in that here we used real time quantitative RT-PCR, whose sensitivity is presumably higher than that of conventional RT-PCR.

Positive ANP, BNP, and CNP immunolabeling was observed in the innermost layer of the human retina, mostly in the internal plexiform layer. The von Willebrand factor antigen signal confirmed that these layers were the most vascularized. The inner and outer nuclear layers also showed ANP, BNP, and CNP labeling. Similar results were reported by Wolfensberger et al. [31] for ANP and BNP in the human retina, though these authors also detected positive ANP-like immunoreactivity in the outer segment of photoreceptors. This may be attributed to the fixation method and the antisera used. In agreement with our results, the presence of ANP was observed in the inner and outer plexiform layers of the rat retina [16].

The presence of all three peptides (ANP, BNP, and CNP) and GFAP noted here in astrocytes suggests their synthesis.
and storage in retinal glial cells, thus playing a role in glial function. The NP systems have been extensively reported in astrocytes. There are reports of ANP and BNP binding on primary astrocytes and glial cell lines leading to cyclic GMP production [32]. Moreover, CNP has been shown to be a potent stimulator of cyclic GMP in cultured mouse astrocytes [33]. ANP immunoreactivity has also been detected in the astrocytes and neurons of the human cerebral cortex, cerebellum, and inferior olivary complex [34]. Interestingly, the finding that glial ANP (mainly in astrocytes) increases in brain infarction [35] led to the suggestion that this peptide could regulate cerebral blood flow, blood-brain barrier permeability, or cerebrospinal fluid volume. Given the similarity of the blood-brain and blood-retinal barriers, future studies will need to establish whether NPs are able to regulate retinal blood flow and blood-retinal barrier permeability.

In the present study, we detected the expression of mRNA corresponding to all three NP receptor subtypes in the human retina. In agreement, Fernandez-Durango et al. [24] demonstrated the presence of mRNA transcripts encoding the three NP receptors (NPRA, NPRB, and NPRC) in the retina, choroid and ciliary process of the rat and rabbit eye by conventional RT-PCR.

Through quantitative real time RT-PCR, we observed that all three NP receptors were expressed in a reproducible pattern in all our specimens. Expression profiles indicated no statistically significant variation in gene expression levels for the NP receptor-encoding transcripts present in human retinal tissue. The NPR mRNA expression has been reported in different tissues. Hohnel et al. [36] showed that, of the three receptors, mRNA expression was highest for NPRA in rat lungs, glomeruli, and left ventricles, followed by the NPRC and NPRB. Moreover, there is evidence of altered NPR numbers on platelets [37], blood vessels [38], the kidney [39] and heart [40] in patients and in animal models with increased circulating ANP concentrations.

In this small sample population, we found no statistically significant correlation between NPR mRNA or β-actin levels and age. Thus, the NP receptor expression pattern seems to remain constant over the age range examined here (13-76 years).

The synthesis of natriuretic peptides and their receptors in the human retina suggests a paracrine or autocrine function. Thus, from a physiological perspective, the presence of ANP and BNP in the RPE indicates that blood-borne NPs may have a significant role in regulating fluid movement across the RPE. This would be analogous to the putative role of NPs in transepithelial ion flux in the choroid plexus [41,42], kidney [43], and colon [44]. Given the similarity of the blood-brain and blood-retinal barriers, we suggest that NPs may be responsible for regulating the volume of the extracellular space in the retina, as has been suggested for the brain [42].

Notwithstanding, given their predominant role in regulating sodium concentrations in other tissues, ANP and BNP may, on the other hand, modulate the distribution of fluid between the intra- and extracellular spaces of the retina. NPs could also act as neurotransmitters or neuromodulators in the retina, as do other peptides such as vasoactive intestinal peptide (VIP) or neuropeptide Y (NPY), which have effects similar to those of ANP [45,46]. Interestingly, it has been suggested that ANP may contribute to cortical spreading depression-induced neuroprotection in rat cerebral cortex, via effects on c-GMP production and other signal-transduction pathways [47]. Thus, it would be of interest to investigate a putative functional role for ANP as a neuroprotecting agent, following a pathophysiological stimulus such as retinal ischemia.

In summary, our findings indicate the expression of NPR transcripts along with that of ANP, BNP, and CNP mRNAs in

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<th>Sample</th>
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<th>NPRB/β-actin</th>
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Relative quantitation by quantitative real time RT-PCR of gene expression of NPR subtypes in the human retina. The NPs expression in human retina was normalized to β-actin expression levels in the same cDNA samples. For the final analysis, total NPR gene expression was calculated and set at 100%. Subsequently, the relative contributions of each receptor subtype as a fraction of this total were determined by calculating the percentage corresponding to the mean-standard deviation of the retina specimens (Figure 4).
the human retina, providing evidence for a local NP system. No differential gene expression levels were detected among the NP receptor-encoding transcripts found in the retina. Real time quantitative RT-PCR served to reliably quantify specific mRNA expression for all three NPRs using small amounts of RNA. Consequently, this method may be useful for evaluating the regulation of this humoral system in ocular diseases such as diabetic retinopathy or glaucoma. We also detected NP expression in retinal neural cells and astrocytes. The expression of NPs in neural, glial, and vascular structures of the normal adult retina, suggest a role in preserving both the neural and vascular integrity of the mature retina. Further studies should address the possible involvement of NPs in ocular diseases.

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