Coexistence of C-Type Natriuretic Peptide and Atrial Natriuretic Peptide Systems in the Bovine Cornea

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Purpose. To determine whether the cornea synthesizes natriuretic peptides and contains their receptors.

Methods. The synthesis of the natriuretic peptides, C-type natriuretic peptide (CNP) and atrial natriuretic peptide (ANP), in the bovine cornea was determined by high-performance liquid chromatography (HPLC) with radioimmunoassay and Southern blot analysis. The presence of natriuretic peptide receptor (NPR)-A and -B and their localizations were measured by reverse transcription–polymerase chain reaction (RT-PCR), in vitro autoradiography, and the activation of particulate guanylyl cyclase by natriuretic peptides in the corneal membrane.

Results. The serial dilution curves of corneal extracts were parallel to the standard curves of CNP and ANP. With reversed-phase HPLC, a major immunoreactive peak of CNP or ANP was observed at the elution time corresponding with synthetic CNP(1-53) or atriopeptin III (AIII), respectively. The presence of mRNAs of CNP and ANP was also detected in the cornea by RT-PCR and/or Southern blot analysis. Production of 3',5'-cyclic guanosine monophosphate (cGMP) by the activation of particulate guanylyl cyclase in the corneal membrane was stimulated by ANP, BNP, and CNP. More cGMP was produced by CNP than by the other natriuretic peptides. Specific 125I-[Tyr]α-CNP(1-22), binding sites were localized in the endothelial cell layer of cornea. The apparent dissociation constant (Kd) value of the cornea was 3.06 ± 0.73 nM and the maximum binding capacity was 3.40 ± 0.63 femtomoles/mm². Both NPR-A and NPR-B mRNAs were detected by RT-PCR.

Conclusions. The cornea synthesizes CNP and ANP and contains their receptors. These results suggest that the CNP and ANP systems coexist in the bovine cornea. (Invest Ophthalmol Vis Sci. 2000;41:2671–2677)
receptors on the corneal endothelium, which may be NPR-C. The synthesis of ANP and the characteristics of its receptor in the cornea are not well-defined. Furthermore, there is no report about the presence of a CNP system in the cornea. The purpose of this study was to define whether the cornea has its own CNP system as well as an ANP system.

**MATERIALS AND METHODS**

**Tissue Collection**

For the measurement of natriuretic peptides (NPs) and GC activity, eyes from healthy adult cattle were obtained from an abattoir within 10 minutes after death and immediately transported to the laboratory in a sterile container. Cornea was separated at 4°C and kept at −70°C until assayed. For the extraction of the mRNAs for NPs and NPRs, cornea was immediately separated at an abattoir, put into liquid nitrogen, and kept at −70°C. For in vitro receptor autoradiography, eyes were immediately snap frozen in isopentane, cooled by dry ice, and stored in sealed boxes at −70°C until sectioned.

**Extraction of NPs and HPLC**

For high-performance liquid chromatography (HPLC), the extraction of CNP was performed as described previously. Briefly, the cornea was boiled in three volumes of distilled water for 10 minutes and then acidified with acetic acid to a final concentration of 0.2 M. The cornea was homogenized (Polytron homogenizer; Brinkman, Westbury, NY), and centrifuged at 25,000g for 30 minutes at 4°C. The supernatant of the tissue homogenate was recentrifuged at 1500g for 40 minutes at 4°C, using a centrifugal concentrator (Centriprep; Amicon, Beverly, MA) to cut high-molecular-weight proteins (over 30 kDa), and then lyophilized. The tissue extract of CNP was reconstituted with 0.1% triffluoroacetic acid (TFA) and subjected to reversed-phase HPLC on an elution (Bondapak; Waters). Elution was performed with a linear gradient of 20% to 60% acetonitrile in 0.1% TFA. The specific activity of [125I]-[Tyr0]-CNP(1-22) was measured by radioimmunoassay (RIA), as described elsewhere. Briefly, the lyophilized samples were reconstituted with phosphate buffer (pH 7.4) containing 50 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.01% sodium azide. After incubation with anti-CNP antibody (Peninsula, Belmont, CA) for 24 hours at 4°C, approximately 15,000 cpm of [125I]-[Tyr0]-CNP(1-22), was added, and samples were incubated again for another 24 hours at 4°C. The separation of the unbound fraction was achieved by the addition of the second antibody. The 50% intercept was at 79.0 ± 14.2 pg/tube (n = 5). The intra- and interassay coefficients of variation were 6.9% (n = 6) and 4.4% (n = 10), respectively. Cross-reactivity with ANP was less than 0.01%, and no cross-reactivity with BNP was observed. RIA for the measurement of ANP was performed as described previously.

**RT-PCR of mRNAs for NPs and NPRs**

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously. Total RNA was extracted from the cornea using TRI reagent (MRC, Cincinnati, OH) according to the manufacturer’s protocol. Total RNA concentrations were quantitated by UV spectrophotometry. One microgram of mRNA was suspended in 20 μl RT buffer containing 10 mM Tris (pH 8.3); 50 mM KCl; 5 mM MgCl₂; 1 mM each of dATP, dCTP, dGTP, and dTTP; 20 U RNase inhibitor; 2.5 μM random hexamers; and 150 U Moloney leukemia virus reverse transcriptase (Perkin Elmer, Branchburg, NJ). mRNA was reverse transcribed at room temperature for 10 minutes and at 42°C for 30 minutes. The reaction was stopped by heat inactivation for 5 minutes at 99°C and then chilled on ice. cDNA products were amplified by PCR with sense and antisense primers.

For CNP, two sets of primers were used: the first round of PCR was performed with a set of CNP primers of large size with 10 μl RT-PCR product as a template. A set of CNP primers of small size was designed within internal sites from the RT-PCR product of CNP primers of large size and was used in the second round of PCR with 5 μl of the first round PCR product. The primer sets were ANP sense 5’-ATGGGCTCCTTCTCTCATCACAAGGGGCTTC-3’ (1-30) and ANP antisense 5’-TTGCAGGCTGGGTCCTCATTGTCA-3’ (7-26) and CNP antisense (small size) 5’-TATATCTGCGGC-3’ (361–380); CNP sense (large size) 5’-CACCAAGGGCTTC-3’ (730–756); and NPR-B antisense 5’-TTAT-CACAGATGCTGTCACAAATCA-3’ (1395–1421). Fifty microliters of PCR buffer contained 10 mM Tris (pH 8.3); 50 mM KCl; 2 mM MgCl₂; 200 μM each of dATP, dCTP,
In Vitro Autoradiographic Binding of

125I-[Tyr0]-CNP(1-22)

Serial 20-μm sections were cut on a cryostat at −20°C, thaw-mounted onto gelatin-chrom-alum-coated slides and dried in a desiccator at 4°C overnight before incubation. The incubation conditions of 125I-[Tyr0]-CNP(1-22) were as previously reported. Briefly, the sections were washed with 150 mM NaCl-0.5% acetic acid (pH 5.0) at room temperature for 10 minutes to remove the endogenous CNP and then preincubated with 30 mM phosphate buffer (pH 7.2) containing 120 mM NaCl and 1 mM phenanthrolene at room temperature for 10 minutes. The sections were incubated with 125I-[Tyr0]-CNP(1-22) in fresh preincubation buffer containing 40 μg/ml bacitracin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.5% BSA at room temperature. The adjacent section was incubated in 125I-[Tyr0]-CNP(1-22) plus 1 μM unlabeled CNP(1-22). After incubation, the sections were washed with fresh preincubation buffer at 4°C for 5 minutes, rinsed three times in cold distilled water at 4°C, and quickly dried under a stream of cold air.

Autoradiographic images were generated by the exposure of the slides with dried 125I-[Tyr0]-CNP(1-22)-labeled eye sections to Hyperfilm-²H (Amersham) in x-ray cassettes together with 20-μm-thick 125I-labeled polymer standard strips (Amersham) at room temperature for 7 days. Autoradiograms were developed (D-19 developer; Kodak) for 3 minutes and fixed in a rapid fixer (Kodak) for 5 minutes at room temperature.

Autoradiographic images were viewed with a macroscope (Wild M 420; Leica, Deerfield, IL) and captured using a video camera (Sony, Tokyo, Japan) with a charge-coupled device iris and a rapid fixer (Kodak) for 5 minutes at room temperature.

Regional bindings of 125I-[Tyr0]-CNP(1-22) in the cornea were analyzed by mean gray-scale value by image analysis software (PRISM ver. 3.6.1; Improved Vision, Coventry, UK). The number of ligand binding sites of different affinities, their apparent dissociation constants (Kd), and their maximal binding capacities (Bmax) on particular structures were derived separately in each individual by Scatchard analysis using an iterative model-fitting computer program (Ligand; Elsevier-BIOSOFT, Cambridge, UK).

Statistical Analysis

Statistical significance of differences was tested using analysis of variance and Dunnett test and the results expressed as mean ± SEM.

RESULTS

Presence of NPs

The presence of irANP in the bovine cornea was demonstrated by a specific and sensitive RIA with HPLC. The serial dilutions of corneal extracts displaced 125I-APIII in a manner parallel to the synthetic standard (Fig. 1A). The concentration of ANP in the cornea was 3.58 ± 0.5 pg/mg of tissue wet weight (n = 6), and the total content of ANP was 1.66 ± 0.25 ng per eye. Figure 1B shows the molecular profile of irANP in corneal extracts by reversed-phase HPLC. Two major immunoreactive peaks were observed: the elution time of major peak corresponded to that of the synthetic APIII. The synthesis of ANP in the cornea was confirmed by RT-PCR. The agarose gel analysis after electrophore-
sis of RT-PCR products is shown in Figure 1C. A band of DNA is present in both atrial and corneal lanes corresponding to the predicted size (364 bp) according to the base pair ladders.

The serial dilution of corneal extracts also displaced $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ in a manner parallel to the synthetic standard (Fig. 2A). The concentration of CNP in the cornea was 0.073 ± 0.004 pg/mg of tissue wet weight ($n = 4$), and the total content of CNP in the eye was 45.9 ± 3.11 pg. The molecular profile of CNP in corneal extracts by reversed-phase HPLC showed a major immunoreactive peak corresponding to synthetic CNP$_{1-53}$ (Fig. 2B). The first round PCR with CNP primers of large size produced a product of the expected size of 571-bp in both pituitary gland and cornea, but other nonspecific products were also amplified from the cornea (data not shown). When the other set of CNP primers was used, the nonspecific bands disappeared. This band was confirmed as CNP by Southern blot analysis (Fig. 2C).

**Particulate GC Activation by NPs**

To determine the presence of NPR-A and NPR-B in the cornea, the activation of particulate GC by NPs in the corneal membrane was measured. The basal rate of cGMP production by particulate GC activation was 3.21 ± 0.36 picomoles/mg protein/min ($n = 6$). By the addition of ANP, BNP, or CNP (10$^{-6}$ M) in the corneal membrane, the rates of cGMP production were 4.30 ± 0.26, 5.00 ± 0.36, or 8.19 ± 0.19 picomoles/mg protein/min, respectively. An increase in cGMP production by CNP, selective ligand to NPR-B, was significantly higher than those by other NPs (Fig. 3). This means that NPR-B may be the predominant NPR in the cornea.

**Autoradiographic Localization of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$-Binding Sites**

Specific $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$-binding sites were demonstrated in the cornea by in vitro autoradiography (Fig. 4). The specifically reversible and strong binding of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ was observed in the corneal endothelium (Fig. 4A). In the presence of 1 μM unlabeled CNP$_{1-22}$, the binding of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ to the corneal endothelium was completely displaced (Fig. 4B). However, the displacement of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ binding to the retina, ciliary body, or iris by 1 μM unlabeled CNP$_{1-22}$ was not observed. Unrelated peptides including angiotensin II or arginine vasopressin (10 μM) did not displace the binding of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ (data not shown).

Analysis of the competitive inhibition of the binding of $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ to the corneal endothelium by increasing the concentrations of unlabeled CNP$_{1-22}$ was consistent with a single high-affinity binding site for $^{125}$I-$[\text{Tyr}^0]-\text{CNP}_{1-22}$ (Fig. 5). The mean $K_d$ and $B_{max}$ values of these sites were 3.06 ± 0.73 nM and 3.40 ± 0.63 femtomole/mm$^2$, respectively.

**Detection of NPR-A and NPR-B mRNAs by RT-PCR**

Figure 6 shows the agarose gel analysis after electrophoresis of RT-PCR products. The positive control for NPR-A was renal medulla and that for NPR-B was pituitary gland. A band of DNA was present in both the positive control and corneal lanes corresponding to the predicted size (451 bp for NPR-A and 692 bp for NPR-B) according to the base pair ladders.

**DISCUSSION**

This study demonstrates for the first time the presence of mRNAs for NPs and NPRs in bovine cornea and the localization of NPR-B in the corneal endothelium.
There are many reports about the presence of ANP in the eye. ANP immunoreactivity has been found in the anterior uvea (31 ng/g tissue), retina (8 ng/g tissue), ANP mRNA was detected in the choroid and ciliary bodies but not in the retina by RT-PCR. However, there has been no systematic study to determine the presence of the NP system in the cornea. In the present study, the concentration of ANP in the bovine cornea was 3.58 ± 0.5 pg/mg tissue, and the total content of ANP was 1.66 ± 0.23 ng. In the molecular profile of corneal irANP, two peaks were found; the major one corresponded to the circulating form of ANP, and the other one was located between APIII and proANP. Stone and Glembotski found that major forms of irANP in the uvea and retina have approximate molecular weights of 2400 and 1750 Da.

\[ \text{Concentration of ANP in the bovine cornea} = 3.58 \pm 0.5 \text{ pg/mg tissue} \]

\[ \text{Total content of ANP} = 1.66 \pm 0.23 \text{ ng} \]

\[ \text{Molecular profile of corneal irANP} \]

\[ \text{Major form} \]

\[ \text{Other form} \]

\[ \text{Molecular weights: 2400 and 1750 Da} \]
respectively, similar in size to the hypothalamic form but clearly distinguishable from the larger cardiac form. We do not know why proANP was not detectable in the cornea. One possibility is the processing of the proANP to the low-molecular-weight form and other fragments during extraction, even though the extraction of ANP was performed in the presence of several protease inhibitors at 4°C after rapid freezing of cornea. However, we found evidence of the synthesis of ANP in the cornea, which was confirmed by the detection of ANP mRNA.

CNP, which is located in vascular endothelium and brain, also is found in other tissues. There is no report about the presence and synthesis of CNP in the eye. Recently, Takashima et al.27 have reported that CNP injected intravitreally causes ocular hypotension. Therefore, CNP may have an important paracrine function similar to ANP. The total content in the cornea was 45.9 ± 3.1 pg, which was 35 times less than the ANP content. We confirmed the synthesis of CNP in the cornea by Southern blot analysis and detection of CNP(1-53). The synthesis of CNP itself in the cornea suggests that it has a paracrine or autocrine function. Therefore, we searched for the presence of biological receptors of NPs in the cornea.

A high density of ANP receptor has been observed on the retina, ciliary body, and cornea. Bianchi et al.16 have reported that the receptor on the epithelium of ciliary body is negatively coupled to adenylyl cyclase and positively coupled to GC. Pang et al.19 have observed ANP receptor in the cultured human trabecular meshwork cells and ciliary muscle cells, in which NPR-B is the primary functional NPR. NPR-A is also found in the retina.17 We found both NPR-A and -B in the cornea. In the corneal membrane, particulate GC activity was markedly induced by the addition of CNP and was more prominent than the activity induced by ANP and BNP. The binding site of CNP was observed in the corneal endothelium. With RT-PCR, the mRNAs for NPR-A and -B were also detected. These results, showing that the major type of biologic receptors in the corneal endothelium is NPR-B, suggest that CNP may be an important hormonal system, even though the level of CNP is low compared with that of ANP. However, Walkenbach et al.22 have reported that ANP receptor in the corneal endothelium is the clearance type and that cGMP is not generated by ANP in corneal endothelial cell culture. The discrepancy in the subtype of NPR in the cornea may be due to the changes in NPR subtypes in the culture system.

The presence of NPs and their receptors in the eye led us to investigate their paracrine actions. In experimental glaucoma, the concentration of ANP in the aqueous humor is increased,21 and the ANP binding sites of ciliary processes are downregulated.14 The physiological role for ANP has been suggested to decrease intraocular pressure in rabbits.14,20,21 Recently, Fernandez-Durango et al.28 have found that CNP is the most potent agent in decreasing intraocular pressure stimulating GC activity in the membrane of rabbit ciliary processes. At present, the physiological significance of ANP and CNP systems in the cornea is unknown. Walkenbach et al.22 have found no ANP effect (1 nM-1 μM) on corneal deturgescence and cGMP production in cultured corneal endothelial cells. From these results, they suggest that corneal endothelial NPR-C may sequester ANP and its metabolites to provide a more constant supply of ANP to the trabecular meshwork.

However, we found the localization of CNP binding sites in the endothelial cells of bovine cornea. The endothelial cells on the inner surface of the cornea are the major cell type responsible for the maintenance of corneal transparency and thickness. The transparency of the cornea is due to its uniform structure, avascularity, and deturgescence. Deturgescence is maintained through passive movement of water out of the cornea.

FIGURE 5. Competitive inhibition curves of specific $^{125}$I-[Tyr$^0$]-CNP(1-22) binding to frozen sections of the bovine cornea. Mean values from five eyes were plotted for the competition of binding of 250 pM $^{125}$I-[Tyr$^0$]-CNP(1-22) to the cornea by increasing concentrations of unlabeled CNP(1-22). Inset: Representative Scatchard plot obtained from one eye of these bovines.

FIGURE 6. Detection of NPR mRNAs by RT-PCR in the cornea (lanes 2, 4), medulla (lane 1), and pituitary gland (lane 3) of the rat as positive control. Lane MM: DNA molecular size marker (174 RF DNA, Hae III cut).
cornea by active ion transport. Although the humoral regulation of this process remains poorly understood, CNP may influence water transport in the endothelial cells. Another role of CNP may be to inhibit the proliferation of corneal endothelial cells. In pathologic conditions in which NPR-B is downregulated, corneal transparency may be disturbed. More studies are needed to define the physiological role of NP systems in the cornea.

Our data suggest that the ANP and CNP systems coexist in the bovine cornea and the NPR-B is the predominant receptor subtype.

References


