Localization of Angiotensin Converting Enzyme in the Ciliary Epithelium of the Rat Eye

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Angiotensin converting enzyme (ACE, E.C. 3.14.5.1) was localized in the rat eye by immunocytochemical staining using anti-rat lung ACE monoclonal antibody, and by autoradiography using the potent ACE inhibitor [3H]captopril. Highest levels of ACE immunoreactivity and [3H]captopril binding were found in the ciliary epithelium (B_max = 1050 fmol [3H]captopril bound/mg protein) while lower levels were present in the sclera (B_max = 500 fmol/mg protein). Scattered regions of the choroidal epithelium were weakly immunocytochemically stained by the anti-ACE antibody. No other major sites of labeling of ACE were demonstrated. In the ciliary epithelium, ACE was confined to cells bordering on the posterior chamber of the eye, similar to its presence at the ventricular surface of the choroidal plexus of the brain. These findings suggest that ACE may metabolize a peptide involved in the regulation of aqueous humor production.

Materials and Methods

[Prolyl-3,4-3H]-S-Acetylcaptofirl (48 Ci/mmol) was provided by NEN:DuPont (Boston, MA), and converted to [3H]captopril with 0.1 M sodium hydroxide as previously described. The production and characterization of the monoclonal antibody A24 to rat lung ACE has been described.

Avidin-biotin-peroxidase complex (ABC) was from Vector Laboratories (Burlingame, CA). The sources of other materials have been described.

Tissue Preparation

Federal guidelines and the ARVO Resolution on the Use of Animals in Research were followed. Adult male Sprague-Dawley rats (200–300 g), anesthetized with sodium pentobarbital, were sequentially perfused intracardially with Hank's balanced salt solution, 0.1% paraformaldehyde with 0.1 M sodium phosphate buffer, pH 7.4, and 0.32 M sucrose in the same buffer. Enucleated eyes were embedded in brain paste and frozen in a dry ice/ethanol slurry. Cryostat
tissue sections (8 μm) were thaw-mounted onto chrome alum/gelatin-coated slides, and stored at -20°C until use.

**Autoradiography**

The binding of [3H]captopril to tissue sections has been described. Briefly, tissue sections were incubated with 4 nM [3H]captopril in the absence or presence of inhibitors in 50 mM Tris-HCl, pH 7.9, 300 mM NaCl, at 4°C for 40 min. Labeled sections were washed twice for 30 sec with the same buffer, dipped in distilled deionized water, and dried rapidly under a stream of cool dry air. Specific binding was defined as the difference between total and nonspecific binding determined in the absence and presence, respectively, of 5 μM enalaprilat, a potent ACE inhibitor. Dried, labeled tissue sections were apposed to LKB Ultrofilm (Uppsala, Sweden) or to Kodak NTB-3 emulsion-coated coverslips for 12 days. Following the development of autoradiograms, the tissue sections were stained with toluidine blue and mounted. The density of silver grains on the autoradiograms was quantitated by computer assisted microdensitometry and converted to fmol [3H]captopril bound/mg protein.

**Biotinylation of ACE Antibody**

Purified anti-ACE antibody A24 (100 μg/ml) was dialyzed against 100 mM sodium borate, pH 8.5, and incubated at 4°C for 24 hr with one-fortieth volume of 1 mg/ml biotinyl-caproyl-N-hydroxysuccinimide in dimethylformamide. The biotinylated antibody was dialyzed against 50 mM Tris-HCl, pH 7.9, at 4°C prior to use in the immunocytochemical studies.

**Immunocytochemical Stain**

Rat eye sections were stained using a modification of the avidin-biotin-peroxidase complex (ABC) technique, as described previously for the localization of ACE in the brain and pituitary gland. Tissue sections were rehydrated, incubated for 30 min at 23°C in periodate-lysine-paraformaldehyde fixative, and washed twice for 20 min with 50 mM Tris-HCl, pH 7.6, 300 mM NaCl. The sections were incubated with biotinylated anti-ACE antibody A24, 500 ng/ml, in 50 mM Tris-HCl, pH 7.9, 300 mM NaCl, 0.1% Triton X-100. After incubation, sections were reacted with ABC reagent, and the peroxidase activity was visualized by incubating the sections in a solution of diaminobenzidine as the substrate. After this reaction was stopped, the sections were treated with 1% osmium tetroxide and 1% potassium ferricyanide in 0.1 M Tris-HCl, pH 7.6, for 5 min. Sections were then stained with toluidine blue and mounted.

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![Fig. 1. Autoradiographic localization of [3H]captopril binding to ACE in the ciliary epithelium. Cryostat tissue sections of rat eye were labeled with [3H]captopril as described in Materials and Methods and apposed to emulsion-coated coverslips. (A) Dark field micrograph of autoradiographic grains appearing over the tissue section shown in (C), which was incubated with 4 nM [3H]captopril. Only the autoradiographic grains, which appear white, are visible. (B) Dark field micrograph of the tissue section shown in (D) incubated with 4 nM [3H]captopril in the presence of 5 μM enalaprilat. (C, D) Bright field micrographs of toluidine blue-stained ciliary epithelium from the rat eye cryostat tissue sections shown in (A) and (B), respectively. Note the high density of labeling over the ciliary epithelium (c), especially at the surface (as indicated by the arrows) bordering the posterior chamber of the eye (pc). Anterior chamber (ac).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933375/)
ton X-100, at 4°C for 36 hr. Bound antibody was detected with 1:200 ABC using hydrogen peroxide and diaminobenzidine as substrates. To verify the specificity of the staining, adjacent sections were incubated with anti-ACE antibody immunoabsorbed with 5 µg/ml of purified rat lung ACE.

Results

[^3H]Captopril Labeling of ACE

[^3H]Captopril bound with high affinity and specificity to the ciliary epithelium and sclera of rat eye (Figs. 1, 2). The binding site for [^3H]captopril in both the sclera and ciliary epithelium was saturable (Fig. 3A). Scatchard analysis indicated a $K_d$ of 2 nM for both tissues (Fig. 3B). This is similar to the affinity of [^3H]captopril for purified ACE. To provide further evidence that [^3H]captopril was labeling ACE in the eye, we examined the potency of several compounds to inhibit ACE binding (Table 1). Enalaprilat, though chemically distinct from captopril, was able to compete for [^3H]captopril binding to eye tissue sections with a $K_i$ of 25 nM. This result agrees with the potency of enalaprilat as an inhibitor of ACE catalytic activity and [^3H]captopril binding to purified ACE, which is chemically similar to captopril but a poor ACE inhibitor, was a weak inhibitor of [^3H]captopril binding to eye sections. EDTA, which blocks ACE activity and [^3H]captopril binding to purified ACE by chelating the active site Zn$^{++}$ ion, also abolished [^3H]captopril binding to eye sections. The similar drug specificity of the [^3H]captopril binding site in the eye and purified ACE indicates that [^3H]captopril specifically labels ACE in the rat eye as in several other tissues.

Localization of [^3H]Captopril Binding in the Rat Eye

Autoradiographic localization of [^3H]captopril binding to rat eye sections demonstrated a high level of ACE in the ciliary epithelium (Fig. 1). At higher magnification, the silver grains were confined to the layer of the epithelium adjacent to the posterior chamber of the eye. Scatchard analysis of [^3H]-

Fig. 2. Distribution of [^3H]captopril binding to ACE in posterior regions of the rat eye. Cryostat tissue sections of rat eyes were labeled with [^3H]captopril as described in Materials and Methods and apposed to emulsion coated coverslips. (A) Dark field micrograph of the autoradiographic grains observed over the tissue section shown in (C). (B) Micrograph of a tissue section incubated with 4 nM [^3H]captopril in the presence of 5 µM enalaprilat. (C, D) Bright field micrograph of toluidine blue stained rat eye tissue sections shown in (A) and (B), respectively. Diffuse specific labeling was observed over the sclera (sc) in (A). No significant specific binding was detectable over the choroid (ch) or the retina (re).
In more posterior regions, the sclera was labeled, but to a lesser degree than the ciliary epithelium (Figs. 2, 3). Specific [3H]captopril binding sites were homogeneously distributed throughout the sclera which demonstrated a B\text{max} of 550 fmol/mg protein upon Scatchard analysis. There was no detectable specific binding to retina (Fig. 2).

Immunocytochemical Localization of ACE in the Eye

To verify the localization of ACE determined autoradiographically by [3H]captopril binding, we employed immunocytochemical techniques to detect ACE histologically. The specificity of the monoclonal anti-rat lung ACE antibody A24 has been described.8,22-23 Binding of biotinylated anti-ACE antibody to rat eye sections was detected using ABC. The localization of the peroxidase reaction products was similar to the localization of silver grains associated with [3H]captopril binding. High levels of immunoreactivity were detected in the ciliary epithelium, particularly at its border on the posterior chamber of the eye (Fig. 4). More posteriorly, staining was localized to the sclera, while the retina was devoid of staining (Fig. 5). Small areas of immunoreactivity were also present in the choroidal epithelium. These sites of ACE immunoreactivity may have been too small or diffuse to be visualized autoradiographically. Staining was abolished from all sites of immunoreactivity when antibody was preabsorbed with purified ACE (Figs. 4, 5).

Discussion

The present study demonstrates high levels of ACE in the ciliary epithelium of the rat eye. The enzyme

Table 1. Specificity of [3H]captopril binding

<table>
<thead>
<tr>
<th>Property</th>
<th>Compound</th>
<th>Ciliary epithelium</th>
<th>Sclera</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD</td>
<td>[3H]Captopril</td>
<td>2 nM</td>
<td>2 nM</td>
</tr>
<tr>
<td>Ki</td>
<td>Enalaprilat</td>
<td>25 nM</td>
<td>30 nM</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>Thiorphan, 5 µM</td>
<td>5%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>EDTA, 1 mM</td>
<td>100%</td>
<td>100%</td>
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Grain densities on autoradiograms of [3H]captopril binding to rat eye sections were quantified for the given regions as described in Materials and Methods. The KD for [3H]captopril is from Figure 3. The Ki for enalaprilat was determined using 4 nM [3H]captopril and 1-1250 nM enalaprilat. Specific binding was determined in eight eye sections at each concentration, and the concentration required for 50% inhibition of [3H]captopril binding was estimated graphically. The Ki was calculated assuming competitive inhibition and a KD for [3H]captopril of 2 nM. The percent inhibition of 4 nM [3H]captopril binding by 1 mM EDTA and 5 µM thiorphan was determined for tissue sections from eight animals.
appears most highly concentrated at the epithelium bordering on the posterior chamber. The level of \(^{[\text{H}]\text{captopril}\) binding in the ciliary epithelium was approximately one-quarter of that in the choroid plexus of the brain, \(^7\) one-half of that in the lung, \(^2\) testis, \(^6\) epididymis and pituitary, \(^3\) and equal to that in the basal ganglia in the brain. \(^7\) Since the ciliary epithelium is strikingly similar to the choroid plexus in structure and function, it may not be surprising that ACE is highly concentrated in both structures.

Although the function of ACE in the ciliary epithelium and choroid plexus of the brain is unclear, some studies have suggested that choroid plexus ACE regulates the level of ventricular A II, \(^3\) which may act on circumventricular organs possessing high levels of A II receptors. \(^3\) A II receptors have not been localized to the choroid plexus. Recently, prorenin, the biosynthetic precursor of renin, was immunocytochemically localized to the nonpigmented epithelium of the ciliary body. \(^8\) Renin cleaves angiotensinogen to A I, which is subsequently cleaved to A II by ACE. The ciliary epithelium has not yet been examined for A II receptors, nor has the aqueous humor been assayed for angiotensin-related peptides. While the ciliary epithelium may form A II to act locally or be transported to more distant targets in the eye, it is also possible that ACE may process other peptide(s) in the eye. ACE isozymes in the male reproductive system \(^4\) and basal ganglia of the brain \(^7\) are thought to act on substrate(s) other than A I. Despite the uncertainty as to the substrate of ciliary epithelial ACE, its specific localization suggests that the enzyme may have a role in regulating aqueous humor production. Further studies with ACE inhibitors currently available may clarify the physiological functions of ACE in the eye.

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Fig. 4. Immunocytochemical localization of ACE in the ciliary epithelium of the rat eye. Fixed cryostat tissue sections were immunocytochemically stained using 500 ng/ml biotinylated monoclonal anti-rat lung ACE antibody A24 as described in Materials and Methods in the absence (A) or presence (B) of purified rat lung ACE. Note ACE immunoreactivity in the ciliary epithelium (c) in (A); intense staining was localized to the surface (indicated by the arrows) bordering the posterior chamber (pc). Anterior chamber (ac).

Fig. 5. Staining of the sclera of rat eye for ACE immunoreactivity. Rat eye sections were immunocytochemically stained using an anti-rat lung ACE antibody as described in Materials and Methods. (A) ACE immunoreactivity in the sclera (sc) and select regions of the choroid epithelium (ch). Staining was absent from the retina (re). (B) Staining was absent when the antibody was preabsorbed with purified rat lung ACE.
Abbreviations

ACE: angiotensin converting enzyme
A I: angiotensin I
A II: angiotensin II.

Key words: ciliary epithelium, angiotensin converting enzyme, kininase II, captopril

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References