Angiotensin-Converting Enzyme Activity in Retinas of Streptozotocin-Induced and Zucker Diabetic Rats
The Effect of Angiotensin II on Na⁺,K⁺-ATPase Activity

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**Purpose.** To investigate whether serum and/or retinal angiotensin-converting enzyme (ACE) activity might correlate with the decrease in sodium potassium adenosine triphosphatase (Na,K-ATPase) activity in the retina of experimentally diabetic rats.

**Methods.** Insulin-dependent diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ) in male Sprague-Dawley rats. Male Zucker fatty diabetic (ZDF/Gmi-fa) rats were used as models of non-insulin-dependent diabetes mellitus. ACE activity in the serum and retina of diabetic rats (1 through 5 months) and age-matched control animals was measured by radioimmunoassay using benzoyl-gly-gly-gly as substrate. The activity of total Na,K-ATPase was determined spectrophotometrically. The α1 and α3 isozymes of Na,K-ATPase were distinguished pharmacologically by their differential sensitivity to ouabain and were measured in the retina.

**Results.** Serum ACE activity was significantly increased in rats with STZ-induced diabetes at 3 weeks through 4 months of diabetes (28% to 32%) but was significantly decreased in ZDF rats after 2 to 5 months of diabetes (—9% to —16%). The activity of ACE in retinas obtained from the same groups of STZ and ZDF rats was significantly reduced at all time points examined in both models (—43% and —55%, respectively). The effect of angiotensin II (AngII) on the activity of Na,K-ATPase in retinas from normal rats was also studied in vitro. AngII significantly lowered the activities of total Na,K-ATPase (—16%) and its α1 and α3 isozymes. The inhibitory effect of AngII was abolished completely by losartan (0.1 μM), a specific antagonist of the AT1 receptor-subtype of AngII, and by nordihydroguaiaretic acid (50 μM), which at this concentration inhibits the lipoxygenase and cytochrome P450-dependent pathways of arachidonic acid metabolism. The inhibitory effect of AngII on the Na,K-ATPase activity was not altered significantly by Nω-iminoethyl ornithine (10 μM), an irreversible nitric oxide synthase inhibitor.

**Conclusions.** The authors suggest that systemic ACE probably is not involved in the mechanisms responsible for the reduced activity of Na,K-ATPase in diabetes. Although AngII inhibits retinal Na,K-ATPase by a mechanism possibly involving arachidonic acid metabolites, it is unlikely that AngII contributes to the decreased Na,K-ATPase activity because of its reduced formation by retinal ACE in diabetes. The possible importance of reduced retinal ACE activity in diabetes warrants further investigation.

Diabetic retinopathy is considered a microvascular disorder present in nearly all patients with insulin-dependent diabetes mellitus (IDDM). Reports indicate that in patients with diabetes, retinopathy is associated with elevated plasma prorenin and angiotensin-converting enzyme (ACE) levels, as well as enhanced prorenin and renin contents in the vitreous fluid compared to subjects without diabetes. Elevated plasma ACE activity also was found in experimentally diabetic animals. These findings have suggested a potential role for the renin-angiotensin system in the development of retinopathy.

The Na⁺ pump is a transmembrane enzyme system that plays a fundamental role in the maintenance...
of cellular integrity. The activity of Na\(^+\)K\(^-\) (Na,K-) adenosine triphosphatase (ATPase) (the enzyme function of the Na\(^+\) pump) is reduced in the retinas as well as in many other tissues of diabetic animals. Recently, we found that the reduced activity of Na,K-ATPase, involving both of its \(\alpha_1\) and \(\alpha_3\) isozymes, is improved in the retina from streptozotocin (STZ) diabetic rats by the long-term administration of captopril, a sulfhydryl ACE inhibitor. The possible relationship between ACE and Na,K-ATPase activity in the retina of diabetic rats has been further examined by measuring serum and retinal ACE levels in streptozotocin-induced diabetic rats, a model for IDDM, and in Zucker diabetic fatty rats, which exhibit characteristics of NIDDM at progressively longer time intervals after production or manifestation of the disease. We also have investigated the effect of AngII in vitro on the activity of total Na,K-ATPase, as well as the \(\alpha_1\) (low ouabain affinity) and \(\alpha_3\) (high ouabain affinity) isozymes of Na,K-ATPase in the normal retina.

**MATERIALS AND METHODS**

**Materials**

Ultrapure chloride salts of calcium, magnesium, potassium, and sodium were obtained from Alfa Products (Danvers, MA). Ile\(^3\)-angiotensin, aprotinin, ATP (Tris-salt, Vanadium free), ethylenediaminetetraacetic acid (EDTA), ethylene-bis(oxethylenenitrilo)tetracetic acid (EGTA), Hanks’ balanced salt solution (HBSS), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes), N\(^2\)-iminoethyl ornithine, nordihydroguaiaretic acid (NDGA), and phenylmethyl-sulfonil fluoride (PMSF) were from Sigma Chemical (St. Louis, MO). Leupeptin and phosphoramidon were purchased from Peninsula Laboratories (Belmont, CA). \(^{[3]}\text{H}\) benzoyl-gly-gly-gly and Ventrex #1 cocktail were purchased from Hycor Biomedical (Ventrex Division, Portland, ME). Protein assay kits were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemical agents were of analytical grade and were purchased from Sigma Chemical.

**Animal Protocols**

All experimental and animal care procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. NIH 85-23, revised 1985) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were housed in standard cages were given standard Rat Chow (Ralston Purina, Richmond, IN) and tap water ad libitum, and they were placed on a 12-hour light–12-hour dark cycle in a room with an ambient temperature of 22°C ± 1°C.

**Induction of IDDM.** Male Sprague–Dawley albino rats (each weighing 150 to 175 g) were purchased from Harlan Sprague–Dawley (Houston, TX). One week after arrival, random groups of eight animals were fasted overnight and were made diabetic by a single intraperitoneal STZ injection (55 mg/kg body weight in 0.05 M citrate buffer, pH 4.5). Age-matched control rats received only the citrate buffer vehicle. Diabetes was verified 3 days later by measurement of nonfasting serum glucose levels in tail vein blood using a glucose oxidase kit (Sigma Chemical). Animals were killed by decapitation at 1, 2, 3, and 4 months after the onset of diabetes (eight rats per age-matched normal and STZ diabetic groups, respectively). Retinas were removed, and blood was collected for serum glucose analysis. All rats treated with STZ had serum glucose concentrations above 350 mg/dl at the time of death.

**Development of NIDDM.** Male Zucker diabetic fatty rats (ZDF/Gmi-fa) and control lean Zucker rats weighing 200 to –250 g were purchased from Genetic Models (Indianapolis, IN). The strain of ZDF rats considered to be a model for type II diabetes was developed in Dr. R. G. Peterson’s laboratory. One, 2, 3, and 4 months after the onset of diabetes, animals were killed (eight rats per group per age), retinas were removed, and blood was collected. The serum glucose of ZDF rats was already above 500 mg/dl after 1 month and was greater than 600 mg/ml 5 months after the onset of diabetes.

**Preparation of Retinal Homogenate for Angiotensin-Converting Enzyme Assay**

Two retinas from the same animal were placed into ice-cold HBSS (pH 7.4), and the vitreous, ciliary body, and retinal pigment epithelium were dissected. Retinas were transferred into 1.5 ml of 0.05 M Hepes buffer (pH 7.4) containing 0.1 M NaCl and 0.1 mM of PMSF and homogenized in a Teflon-glass homogenizer (Thomas Scientific, Swedesboro, NJ) using 20 strokes. Aliquots of the homogenate were used for ACE determinations.

**Angiotensin-Converting Enzyme Assay**

Angiotensin-converting enzyme activity was determined according to Erdős and coworkers. The specificity of the reaction was confirmed by including 1.5 \(\mu\)M captopril in parallel assays. The 150 \(\mu\)l of assay mixture contained 0.05 M Hepes, 0.1 M NaCl, 0.6 M Na\(_2\)SO\(_4\) (pH 7.4), 0.1 mM PMSF, 25 \(\mu\)M leupeptin, and 10 \(\mu\)M aprotinin protease inhibitors, benzoyl-gly-gly-gly as substrate (in a final concentration of 5 mM containing \(10^5\) cpm activity) and 50 \(\mu\)l of retinal homogenate. The reaction was initiated by the addition of ACE substrate and was allowed to proceed for 2 hours at 37°C. The reaction was stopped by 50 \(\mu\)l 0.5
N HCl. [3H]benzoyl-gly released was extracted with Ventrex #1 cocktail and counted in an LS 9000 liquid scintillation counter (Beckman Instruments, Fullerton, CA) for 10 minutes. Results are expressed as pmol benzoyl-gly released/hour⁻¹⋅mg protein⁻¹ ± SEM.

For ACE measurements, serum samples were diluted 10 times with assay buffer. The 150 μl of assay mixture contained 0.05 M Hepes buffer without protease inhibitors, 50 μl of diluted serum sample, and radiolabeled substrate. The ACE activity is expressed as pmol benzoyl-gly released/hour⁻¹⋅mg protein⁻¹ ± SEM.

Incubation of Normal Retinas With AngII

The effect of AngII (1 nM to 1 μM) was studied on the activity of total retinal Na,K-ATPase and its α1 and α3 isozymes. Retinas were removed quickly and placed in ice-cold Tris-EGTA buffer (30 mM Tris-base, 130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM EGTA), pH 7.4, 305 ± 5 mOsm as determined by a Wescor (Logan, UT) 5100C vapor pressure osmometer. Exogenous tissue was removed, and the retinas from each rat were transferred individually into 5 ml HBSS (pH 7.4) containing 2 mM CaCl₂, 2 mM MgCl₂, and 1 μM phosphoramidon, an inhibitor of neutral endopeptidase (E.C. 3.4.24.11). After equilibration for 10 minutes at 37°C, AngII was added and incubation was continued for 10 minutes.

Preparation of Retinal Homogenate for Na,K-ATPase Assay.

After incubation, each retina was homogenized in 900 μl of ice-cold 1 mM EDTA-Tris buffer (pH 7.4) in a Teflon-glass homogenizer (Thomas Scientific, Swedesboro, NJ) using 20 strokes. After removing aliquots for protein assay, 870 μl of 1 mM EDTA-Tris buffer (pH, 7.4) containing 0.6 mg/ml sodium dodecyl sulfate and 1% bovine serum albumin was added, and the retinal homogenate was incubated for 10 minutes at room temperature. Aliquots of the homogenate were used for ATPase determinations.

Na,K-ATPase assay

Na,K-ATPase activity was determined in retinal homogenates from normal and diabetic rats by determining spectrophotometrically the inorganic phosphate (P₁) liberated from ATP as previously described. The assay mixture contained 130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM ATP, 1 mM EGTA, 30 mM Tris-HCl (pH 7.7), 0.1 ml homogenate and 0.05 M of protein in either the absence or presence of ouabain (5 × 10⁻⁸ M to 10⁻³ M) in a final volume of 0.5 ml. The activities of the α1 and α3 isozymes of Na,K-ATPase were determined by measurements in the presence of their half-maximal inhibitory concentrations (IC₅₀) of ouabain (2 × 10⁻⁷ M and 5 × 10⁻⁸ M, respectively). The reaction was initiated by the addition of ATP and was allowed to proceed for 30 minutes at 37°C. Total Na,K-ATPase activity was taken as the difference between the activities recorded in the absence and presence of 10⁻³ M ouabain. Enzyme-specific activities are expressed as μmol P₁/hour⁻¹⋅mg protein⁻¹ ± SEM.

Protein Assay

Protein was estimated by Coomassie blue G binding using bovine serum albumin as a standard.

Data Analysis

Data are presented as mean ± SEM of five to eight separate experiments performed using triplicate sam-
RESULTS

Activities of Serum and Retinal Angiotensin-Converting Enzyme of STZ Diabetic and ZDF Rats

The general condition of STZ diabetic and ZDF rats was assessed by determining final body weights, blood glucose levels, and HbA1c. Results are shown in Table 1. STZ rats displayed significantly reduced growth, reflected in their lower body weight, compared to age-matched controls. Blood glucose and HbA1c levels were three to five times greater in diabetic animals than in age-matched controls.

Angiotensin-converting enzyme activity in serum from STZ diabetic rats was significantly elevated (28% to 32%) compared to those for age-matched normal at 1 through 4 months after the induction of diabetes. The highest serum ACE activity was measured at 2 months of diabetes. Thereafter, the enzyme activity gradually declined, but it remained significantly greater than control values at all time points (Fig. 1A). In contrast, ACE activity in serum from ZDF rats was significantly decreased from the second month through the fifth month of diabetes compared to age-matched lean controls (−9%, −17%, and −16%, respectively) (Fig. 1B). The ACE activity in the serum of age-matched normal rats was significantly greater for Zucker lean rats than for Sprague–Dawley rats.

Angiotensin-converting enzyme activity also was measured in the serum of rats injected with a high dose of STZ and considered to have severe, poorly controlled diabetes managed by minimal insulin treatment. The serum ACE activity in these rats also was increased significantly in the serum by 23% and was reduced significantly in the retina (−30%) 3 months after the induction of diabetes (data not shown).

The ACE activity in retinas obtained from 2- and 4-month-old STZ diabetic rats was decreased substantially (−38% and −43%, respectively) compared to corresponding values for age-matched normal animals (Fig.
FIGURE 2. Angiotensin-converting enzyme activity in the retina of STZ and ZDF rats. Enzyme-specific activities are expressed as pmol [3H]bz-gly/hour⁻¹·mg protein⁻¹. (A) Streptozotocin-induced (STZ) rats and control. (B) Zucker diabetic fatty rats (ZDF) and lean controls. Values are mean ± SEM of triplicate samples. *P < 0.001 difference versus control.

Effect of AngII on the Activity of Total Na,K-ATPase and Its α1 and α3 Isozymes in the Normal Retina

Total Na,K-ATPase and component isozyme activities were assayed in retinas preincubated in HBSS medium in the absence and presence of AngII (1 nM to 100 nM). Isozyme activities measured in the presence of the appropriate IC₅₀ of ouabain are shown in Table 2. The addition of 100 nM AngII significantly inhibited the total Na,K-ATPase activity by 16% (Fig. 3). The inhibitory effect of 100 nM AngII was abolished completely when retinas were preincubated with for 10 minutes with 0.1 μM losartan, a specific antagonist for the AT1 subtype of the AngII receptor. Exposure of retinas to AngII significantly inhibited both the α1 and α3 Na,K-ATPase isozymes. The α1 isozyme was more sensitive to AngII inhibition because its activity was significantly reduced in the presence of 1 nM AngII, whereas the α3 isozyme was only decreased by 10 nM AngII or greater (Table 2).

To assess the possible involvement of nitric oxide (NO) synthase in the mechanism of action of AngII, retinas were incubated in the medium containing 10 μM N⁵-iminoethyl ornithine (L-NIO), an irreversible inhibitor of NO synthase. L-NIO by itself had no discernible effect on the inhibition of total Na,K-ATPase activity by AngII (Fig. 3). To investigate the possible involvement of arachidonic acid metabolites in the mechanism of action of AngII, retinas were incubated in medium containing 50 μM NDGA, which inhibits the lipoygenase pathway and cytochrome P-450-dependent arachidonic acid metabolism. The inhibitory effect of AngII on the activity of total Na,K-ATPase and each of its isozymes was abolished completely by NDGA, whereas this agent alone had no effect (Fig. 3; Table 2).

DISCUSSION

In the current study, the activity of ACE was found to be increased in the serum and decreased in the retina of STZ-treated Sprague-Dawley rats used as models of IDDM. Serum ACE levels were elevated consistently through 4 months of diabetes. Previously, we found similar alterations of ACE levels in the serum and retina of Long-Evans rats after 2 months of STZ administration. As reported earlier, the activity of total Na,K-ATPase and its α1 and α3 isozymes are decreased after
TABLE 2. Effect of Angiotensin II on the Activities of the α1 and α3 Isozymes of Na,K-ATPase in Normal Retinas

<table>
<thead>
<tr>
<th>Group</th>
<th>α1 Na,KATPase* (µmol P· hour⁻¹ · mg protein⁻¹)</th>
<th>P</th>
<th>α3 Na,KATPase* (µmol P· hour⁻¹ · mg protein⁻¹)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AngII</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>8</td>
<td>3.43 ± 0.11</td>
<td>&lt;0.001</td>
<td>10.45 ± 0.24</td>
</tr>
<tr>
<td>100 nM</td>
<td>10</td>
<td>3.30 ± 0.13</td>
<td>&lt;0.001</td>
<td>10.38 ± 0.13</td>
</tr>
<tr>
<td>10 nM</td>
<td>6</td>
<td>2.90 ± 0.90</td>
<td>&lt;0.0001</td>
<td>10.71 ± 0.31</td>
</tr>
<tr>
<td>LOS 0.1 µM</td>
<td>8</td>
<td>3.52 ± 0.19</td>
<td>&lt;0.001</td>
<td>12.11 ± 0.36</td>
</tr>
<tr>
<td>LOS + AngII 100 nM</td>
<td>8</td>
<td>4.24 ± 0.13</td>
<td>NS</td>
<td>12.00 ± 0.27</td>
</tr>
<tr>
<td>L-NIO 10 µM</td>
<td>8</td>
<td>4.16 ± 0.08</td>
<td>NS</td>
<td>12.06 ± 0.26</td>
</tr>
<tr>
<td>L-NIO + AngII 100 nM</td>
<td>8</td>
<td>4.21 ± 0.16</td>
<td>NS</td>
<td>13.05 ± 0.35</td>
</tr>
<tr>
<td>NDGA 50 µM</td>
<td>7</td>
<td>4.13 ± 0.09</td>
<td>NS</td>
<td>13.08 ± 0.19</td>
</tr>
<tr>
<td>NDGA + AngII 100 nM</td>
<td>7</td>
<td>4.05 ± 0.13</td>
<td>NS</td>
<td>12.66 ± 0.15</td>
</tr>
</tbody>
</table>

* The activities of α1 and α3 isozymes were measured in the presence of the IC₅₀ of ouabain (2 × 10⁻⁵ M and 5 × 10⁻⁶ M, respectively). Retinas were preincubated in the presence of LOS (losartan), L-NIO (N°-iminoethyl ornithine), or NDGA (nordihydroguaiaretic acid) for 10 minutes before the addition of angiotensin II (AngII) to the incubation medium. NS = not significant.

2 months of STZ diabetes. In contrast, ZDF rats, models of NIDDM, exhibited decreased serum ACE activity compared to lean controls after 2 months of diabetes, and this further declined through 5 months of diabetes. ZDF rats, like STZ diabetic animals, also exhibited decreased ACE activity in the retina. The opposite changes in serum ACE levels in the two diabetic models suggest that circulating ACE probably is not involved in the mechanism responsible for the impaired Na,K-ATPase activity in the diabetic retina.

Angiotensin-converting enzyme activity decreased more markedly in the retinas of ZDF rats than in those of STZ rats after 2 months of diabetes and remained depressed for an additional 3 months. Previously we found the total Na,K-ATPase activity in the retinas of ZDF rats already reduced after 1 month of diabetes, a decline that manifested itself 1 month earlier than that observed in STZ rats. In addition, the activities of total Na,K-ATPase and its isozymes are substantially more diminished in ZDF rats than in STZ rats. Although a direct relationship between the Na⁺ pump and ACE activity has not been demonstrated, it has been suggested that the ACE activity in bovine endothelial cells in culture is under cation regulation and may be modulated by ouabain-sensitive Na,K-ATPase. Endothelial ACE activity is decreased significantly by low concentrations of ouabain and is activated by calcium and sodium ionophores. Recently, we found that the reduced activity of retinal Na,K-ATPase of STZ diabetic rats is ameliorated by the long-term administration of captopril, a sulfhydryl ACE inhibitor by an unknown mechanism.

The presence of ACE, AngII, and AngII-binding sites in the retina, as well as the finding of a vasoconstrictor effect of locally infused AngII, point to a role of the tissue renin–angiotensin system in the control retinal blood circulation. Our results suggest that the potential for AngII formation by ACE in the retina of diabetic rats is reduced substantially. Decreased AngII formation...
could be one of the factors that result in dilated vessels and capillary hypertension in early diabetic complications. Further, recent reports strongly indicate neuromodulatory effects of the local renin–angiotensin system within the retinal circuitry. The hypothesis that AngII exerts direct neuromodulatory action independent of its vasoactive effect is supported by immunohistochemical studies indicating that the highest concentrations of ACE were detected in the neurons of the proximal and distal retina, and not in the retinal vascular components. These findings suggest that the neuromodulatory effect of AngII also might be diminished in diabetes.

Our results demonstrate that AngII significantly inhibits the total activity of retinal Na,K-ATPase, as well as that of its α1 and α3 isozymes. The inhibitory effect of AngII is receptor mediated because it was abolished by losartan, a specific inhibitor of the AT1 receptor subtype. The NO synthase inhibitor L-NIO did not antagonize the inhibition exerted by AngII under our experimental conditions, suggesting that NO is not involved in modulating the AngII effect. However, our findings are consistent with the involvement of unidentified arachidonic acid metabolites because NDGA, an inhibitor of the lipoxigenase and cytochrome P-450 pathways of arachidonic acid metabolism, completely abolished the AngII inhibition of total Na,K-ATPase and its component isozymes.

A clear-cut role of AngII as a possible modulator of the Na+ pump must be defined because contradictory and limited reports have been published. Our data demonstrate that the activity of the α1 isozyme is inhibited more readily by AngII than that of the α3 isozyme. AngII might contribute to the maintenance of ion gradients in neural elements and in cellular components of microvessels under physiological conditions. Previous reports indicate that AngII stimulates the Na+-H+ antiporter in vascular smooth muscle cells by the activation of protein kinase C and in the proximal convoluting tubules of the kidney by two mechanisms, the G-coupled reduction in intracellular adenosine 3',5'-cyclic monophosphate and the activation of protein kinase C. Although protein kinase C stimulates the Na+-H+ antiporter, it also reduces, Na,K-ATPase. Thus, the inhibitory effect of AngII on the retinal Na,K-ATPase also might be mediated by the stimulation of protein kinase C.

In contrast to our results obtained with the retina, AngII was found to stimulate ouabain-inhibitable Na+ pump activity in renal cortical microsomal preparations, in cultured aortic smooth muscle cells, and in aortic endothelium-smooth muscle ring preparations. This raises the possibility that AngII might act by different pathways in different tissues.

Key Words
angiotensin, angiotensin-converting enzyme (ACE), Na+,K+-ATPase, retina, streptozotocin-diabetes

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References


