Angiotensin II receptors are expressed and functional in human esophageal mucosa

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IT IS WELL KNOWN FROM TEXTBOOKS in physiology that the renin-angiotensin system (RAS) is a prominent regulator of body fluid balance and blood circulation. Only a few studies have been devoted to the actions of the renin-angiotensin system in the human gastrointestinal tract. The present study was undertaken to elucidate the expression and action of RAS in the human esophageal mucosa. Mucosal specimens with normal histological appearance were obtained from healthy subjects undergoing endoscopy and from patients undergoing esophagectomy due to neoplasm. Gene and protein expressions of angiotensin II (Ang II) receptor type 1 (AT1) and type 2 (AT2) and angiotensin-converting enzyme (ACE) were analyzed. In vivo functionality in healthy volunteers was reflected by assessing transmucosal potential difference (PD). Ussing chamber technique was used to analyze the different effects of Ang II on its AT1 and AT2 receptors. Immunoreactivity to AT1 and AT2 was localized to stratum superficiale and spinosum in the epithelium. ACE, AT1, and AT2 were found in blood vessel walls. Transmucosal PD in vivo increased following administration of the AT1 receptor antagonist candesartan. In Ussing preparations mean basal transmural PD was −6.4 mV, epithelial current (Iep) 34 μA/cm², and epithelial resistance (Rep) 321 Ω·cm². Serosal exposure to Ang II increased PD as a result of increased Iep, whereas Rep was constant. Ang II given together with the selective AT1-receptor antagonist losartan, or AT2 agonist C21 given alone, resulted in a similar effect. Ang II given in presence of the AT2-receptor antagonist PD123319 did not influence PD, but Iep decreased and Rep increased. In conclusion, Ang II receptors and ACE are expressed in the human esophageal epithelium. The results suggest that AT2-receptor stimulation increases epithelial ion transport, whereas the AT1-receptor inhibits ion transport and increases Rep.

potential difference; epithelial current; epithelial resistance

MATERIALS AND METHODS

Subjects and Tissue Specimens

Biopsies for expression analyses. Healthy subjects (n = 8, mean age 31 yr, range 25–62 yr, 1 woman) with no history of gastrointestinal disease volunteered to esophagogastrectomy with mucosal biopsy takings. In general, three biopsies were taken in the distal esophagus 2 cm above the gastroesophageal junction (Z line) and were used for histology, immunohistochemistry, Western blotting, and RT-PCR. Only subjects with normal mucosal appearance at endoscopy and at histological examination were included. Resected tissues (see below) were also used for expression analyses.

Mucosal specimens for functional analysis in vitro. For in vitro analysis using the Ussing chamber technique, mucosal specimens from the region distal to the midesophagus were obtained from 17 patients (mean age 62.2 yr, range 46–80 yr, 4 women) undergoing radical surgery for malignancy at the esophagogastric junction. Since all specimens were obtained from patients with a neoplastic process, care was taken not to include tissue close to the pathological process. In addition to normal macroscopic appearance, the resected mucosal specimens used in Ussing chamber experiments were also confirmed normal by histology.

In vivo investigation. Thirteen healthy volunteers (mean age 30 yr, range 23–62 yr, 2 women) participated in the functional examinations. All subjects were without history of gastrointestinal disease and were Helicobacter pylori negative as tested by 13C-urea breath test.

Ethics

The study was approved by the University of Gothenburg Ethics Committee as well as by the Regional Ethical Review Board in Gothenburg and was performed in accordance with the Declaration of
Helsinki. All participants taking part in the study were informed verbally as well as in writing and signed a consent form before inclusion.

Histology

Both endoscopic biopsies and samples of the resected mucosal specimens were fixed in buffered 4% formaldehyde, dehydrated, and embedded in paraffin. For evaluation of general morphology, 3-μm sections were mounted on slides and stained with hematoxylin-eosin.

Immunohistochemistry

Sections for immunohistochemistry were deparaffinized and then boiled for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The Immunocruz Staining System (Santa Cruz Biotechnology, Santa Cruz, CA) was used for the immunohistochemistry protocol. After inhibition of endogenous peroxidase activity, the slides were preincubated with serum block and then incubated with primary antibodies against ACE and AT1 and AT2 receptors over 2 h in room temperature in dilutions of 1:100 (see Table 1). The primary antibody was raised in goat, rabbit, and goat, respectively (Santa Cruz Biotechnology). Control sections were incubated with normal goat or rabbit IgG 0.4 μg/μl instead of the primary antibody. After being washed, the slides were incubated with biotinylated secondary antibody and the complex was detected by use of horseradish peroxidase-streptavidin. The color was developed using 3,3′-diaminobenzidine.

Western Blot Analyses

Specimens were snap frozen in liquid nitrogen and kept frozen for later Western blot analysis of ACE and AT1 and AT2 receptor expressions. The frozen specimens were sonicated in a PE buffer (10 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics, Stockholm, Sweden). The homogenate was then centrifuged (10,000 g for 10 min at 4°C) and the supernatant was analyzed for protein content by the Bradford method and stored at −80°C (4). Samples were diluted in SDS buffer and heated at 70°C for 10 min before they were loaded on a NuPage 10% Bis-Tris gel, and electrophoresis was performed using the FastStart DNA Master SYBR Green I (Roche Diagnostics). PCR was performed containing 2 μl of each RT sample by the hot-start technique. MgCl2 concentration was optimized to 4 mM to obtain the highest signal intensity and lowest background. For each tested sample the copy number of the PCR products was calculated by dividing these values by the total RNA concentration as an internal standard, measured by use of a GENios spectrophotometer (TECAN, Salzburg, Austria). Copy numbers of target genes were then expressed as copy number per microgram total RNA (27). The quantification was carried out by the software supplied by Roche Diagnostics (Mannheim, Germany). The primer sequences, PCR products sizes, and references are listed in Table 1.

Table 1. RT-PCR-related information and antibodies and positive controls used in Western blot and immunohistochemistry

<table>
<thead>
<tr>
<th>RT-PCR</th>
<th>Primer sequences</th>
<th>Size, bp</th>
<th>Reference</th>
<th>Western Blot and IHC Primary Antibody</th>
<th>Western Blot Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>F: 5’-ccgatctcgasgcagcctc-3’</td>
<td>408</td>
<td>13</td>
<td>N-20</td>
<td>Kidney extract</td>
</tr>
<tr>
<td></td>
<td>R: 5’-gtgctccagtcgctccc-3’</td>
<td></td>
<td></td>
<td>sc-12184</td>
<td>Sc-2255</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>F: 5’-ggccagtctcttctttctgtatgacac-3’</td>
<td>210</td>
<td>17</td>
<td>N-10</td>
<td>PC-12</td>
</tr>
<tr>
<td></td>
<td>R: 5’-tgacatagctggaggtatgatcaatgc-3’</td>
<td></td>
<td></td>
<td>sc-1173</td>
<td>Sc-2250</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>F: 5’-gtctcccttctttgtatgct-3’</td>
<td>253</td>
<td>17</td>
<td>N-19</td>
<td>HepG2</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ctctctcaggacttagtggtc-3’</td>
<td></td>
<td></td>
<td>sc-7421</td>
<td>Sc-2227</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; ACE, angiotensin-converting enzyme; AT1 and AT2, angiotensin II receptor types 1 and 2, respectively; F, forward; R, reverse.
antagonist candesartan (16 mg) ingested at 2200 the evening before the experiment. This time and dose was chosen on the basis of previous reports on plasma concentration and functional AT₁ receptor interference, the latter assessed as effects on renin-release (2, 11). After intubations and identification of the high-pressure zone as well as the PD step-up, the subjects were left for 30 min to allow basal conditions to be established. A modified Bernstein-test was then performed by infusion of 100 mM HCl + 50 mM NaCl (the latter to achieve isotonicity) into the midesophagus at 2 ml/min over 20 min followed by 150 mM NaCl at 2 ml/min over 10 min. As reported elsewhere this experimental setup was used also for evaluating primary peristalsis and transient lower esophageal sphincter relaxations (6). In the present study the transmucosal PD values recorded 2 cm oral to the high-pressure zone (the manometric reflection of the lower esophageal sphincter) were analyzed during baseline conditions and during luminal acidification (both situations with manometrically confirmed absence of peristaltic motor activity).

**Ussing Chamber Experiments**

The resected esophageal specimens were immediately immersed in ice-cold oxygenated (95% O₂ and 5% CO₂) Krebs solution with the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose and transported to the laboratory. The esophageal mucosa was gently removed from the muscular layers by sharp dissection during superfusion with cold oxygenated Krebs solution and was then mounted in a standard vertical Ussing chamber with a 4 × 8 mm oblong opening (Warner Instruments, Hamden, CT). After mounting, each half chamber was filled with 5 ml Krebs solution, bathing both the mucosal and serosal side of the specimen. The Krebs solution was maintained at 37°C and continuously oxygenated with 95% O₂ and 5% CO₂ and stirred by gas flow in the chambers. Depending on the size of the resected material 2–4 preparations could be retrieved from a single individual. PD was measured with a pair of matched calomel electrodes (REF401, Radiometer Analytical, Copenhagen, Denmark) and the epithelial electrical resistance (Rₑp) was assessed by use of the Ussing pulse method. The latter has the advantage of specifically estimating Rₑp and is described in detail elsewhere (22), for which reason only a brief description is given here: The total resistance in an Ussing chamber is dependent on Rₑp as well as other in-series coupled electrical resistances, for example in subepithelial tissue that below will be termed “external resistance” (Rₑx). The Ussing pulse method is based on the fact that the electrical behavior of an epithelium corresponds to a resistor and capacitor in parallel and that active ion transport is to be regarded as a generator of epithelial current (Iₑp) (10, 12). To assess Rₑp, an electrical square-wave current pulse is sent across the mucosal preparation in the Ussing chamber and the voltage response is measured. The pulse character is individualized for each preparation to be between 20 and 100 µA with duration of 1 ms to obtain a voltage deflection, preferably between 50 and 100 mV, optimal for analysis as well as an electrical field without significant influence on epithelial functions. Because the epithelium, unlike the rest of the tissue and surrounding baths, has capacitor property (thus in parallel with the electrical resistance of the epithelium; Rₑp) the

![Image](http://ajpgi.physiology.org/obj/c1020333.jpg)

Fig. 1. Human esophageal mucosa at ×40 magnification with brown indicating positive immunostaining. A: immunostaining for angiotensin-converting enzyme (ACE) was localized in the blood vessel walls in the lamina propria (see arrow). B: staining for the angiotensin II type 1 (AT₁) receptor in the epithelium is most obvious in stratum superficiale and spinosum. C: angiotensin II type 2 (AT₂) receptor staining is most obvious in stratum superficiale and spinosum. D: negative control for the AT₂ receptor. E: esophageal mucosa in C and D stained with hematoxylin-eosin.
RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>ACE</th>
<th>AT1 receptor</th>
<th>AT2 receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoscopy (n = 8)</td>
<td>0.0 (0–5.65)</td>
<td>0.0 (0–9.0)</td>
<td>0.089 (0.003–2.96)</td>
</tr>
<tr>
<td>Resection (n = 7)</td>
<td>1.92 (0.06–28.4)</td>
<td>0.136</td>
<td>0.823</td>
</tr>
</tbody>
</table>

Western Blot

<table>
<thead>
<tr>
<th></th>
<th>Endoscopy (n = 7)</th>
<th>Resection (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>5.7 (2.8–11.4)</td>
<td>6.0 (0–10.0)</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>95.1 (47.7–226.3)</td>
<td>76.0 (74.7–100.7)</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>56.1 (25.5–78.1)</td>
<td>45.9 (21.8–61.2)</td>
</tr>
</tbody>
</table>

Table 2: Expression of gene transcripts and proteins related to RAS in esophageal mucosa retrieved by endoscopic biopsy-takings and surgical resection

**Drugs and Chemicals**

The following chemicals were used in the in vitro study: Ang II; PD123319 and amiloride (Sigma Chemical, St. Louis, MO); losartan (Merck); and C21 (gift from Dr. A. Hallberg, Department of Medicinal Chemistry, Uppsala University, Uppsala, Sweden), were all dissolved in Krebs solution. In the in vivo study the AT1 receptor antagonist candesartan (Atacand, AstraZeneca, Mölndal, Sweden) was used (dose and administration given above).

**RESULTS**

**Expression of RAS in Human Esophageal Mucosa**

Immunohistochemistry performed on endoscopically retrieved mucosal biopsies and resected tissue revealed a distinct staining for ACE in the capillary walls located at the tip of the papillae and in the blood vessel walls in the lamina propria (Fig. 1A). Immunostaining for AT1 receptors was detected in the epithelium and most obvious in stratum superficiale and spinosum (Fig. 1B). AT1 receptor immunoreactivity was also detected in the blood vessel walls supplying the epithelium and in the lamina propria. Immunostaining for AT2 receptors was detected in the epithelium and, similar to AT1 receptor, most distinctly in stratum superficiale and spinosum as well as in blood vessel walls supplying the epithelium in the lamina propria (Fig. 1C). In both cases the lamina propria papillae were negative. Negative controls remained unstained in all specimens (example given in Fig. 1D). Western blotting exhibited a strong AT1 and AT2 receptor immunoreac-
tivity (Fig. 2, A and B), whereas only a faint staining of ACE was detected in the esophageal mucosal tissue samples (Fig. 2C). Expressions of gene transcripts and proteins, respectively, were of similar order of magnitude independent of whether the biopsies were retrieved from endoscopic investigations or from resected esophagii (Table 2).

Transmucosal PD Measurements In Vivo

By use of the pressure profile obtained from the intraesophageal manometric catheter the high-pressure zone (hpz) corresponding to the lower esophageal sphincter was identified. PD values at a level 2 cm oral to the hpz were used for analysis. Baseline esophageal PD values differed markedly between individuals, ranging from \(-3.5\) to \(-30\) mV. The PD values after intake of the AT1 receptor antagonist candesartan were significantly higher (more lumen negative) compared with control level \((P < 0.05)\) (Fig. 3A).

Luminal perfusion with hydrochloric acid (HCl, 100 mM with NaCl 50 mM to obtain isotonicity) elicited a transient increase in PD with a peak appearing after 4–5 min. PD then slowly declined to reach baseline values after 15–20 min. The delta PD response (peak minus baseline) to HCl perfusion was significantly larger after intake of candesartan compared with controls \((P = 0.032)\) (Table 3). Because both baseline PD and the HCl-induced PD change increased, it follows that the acid-induced peak PD was higher in all except one case after candesartan administration \((P = 0.013)\) (Fig. 3B).

In Vitro Analysis Using the Ussing Chamber Technique

Time control and baseline. After mounting and a 30- to 45-min equilibration period, the prepared mucosa remained electrically stable for upward of 2 h \((n = 2)\). In the pharmacological experiments baseline values of PD, \(R_{ep}\), and \(I_{ep}\) were measured over 30 min, after which Ang II receptor blocking agents (losartan or PD123319) or vehicle were added. Neither of the two blocking agents significantly influenced baseline values, as shown in Table 3. After another 15 min the agonists Ang II or C21 were given stepwise as described in MATERIALS AND METHODS.

Effects of Ang II and interference with the subreceptor. Ang II added to the serosal side in the chamber caused a

Table 3. Baseline electrical parameters in the human esophageal mucosa mounted in Ussing chambers

<table>
<thead>
<tr>
<th></th>
<th>PD (mV)</th>
<th>Postdrug, % change</th>
<th>(P) value</th>
<th>(R_{ep}) (ohm cm(^2))</th>
<th>Postdrug, % change</th>
<th>(P) value</th>
<th>(I_{ep}) ((\mu A/cm^2))</th>
<th>Postdrug, % change</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ((n = 14, N = 16))</td>
<td>6.4±0.4</td>
<td>2.7±1.9</td>
<td>0.17</td>
<td>321±23</td>
<td>1.4±2.1</td>
<td>0.46</td>
<td>34.5±3.8</td>
<td>2.8±2.5</td>
<td>0.88</td>
</tr>
<tr>
<td>Losartan ((n = 5, N = 6))</td>
<td>7.2±0.6</td>
<td>5.5±6.3</td>
<td>0.34</td>
<td>381±78</td>
<td>0.2±2.1</td>
<td>0.22</td>
<td>32.6±4.9</td>
<td>6.0±6.6</td>
<td>0.75</td>
</tr>
<tr>
<td>PD123319 ((n = 6, N = 10))</td>
<td>6.3±0.4</td>
<td>5.3±2.7</td>
<td>0.11</td>
<td>340±25</td>
<td>6.2±4.2</td>
<td>0.09</td>
<td>25.9±2.1</td>
<td>3.8±5.9</td>
<td>0.88</td>
</tr>
</tbody>
</table>

The postdrug represents the value 15 min after drug addition. Values are given as means ± SE (range); \(n\), number of patients; \(N\), number of preparations. PD, potential difference; \(R_{ep}\), epithelial resistance; \(I_{ep}\), epithelial current.
concentration-dependent rise of both PD and $I_{ep}$, with maximal values ~20% above baseline ($P = 0.001$ and 0.003, respectively), whereas no significant effect on $R_{ep}$ was observed (Fig. 4).

Ang II given in presence of the AT$_1$ receptor antagonist losartan $10^{-6}$ M induced a response pattern that did not differ from when Ang II was given alone: PD increased 20% (pEC50 for control 9.59 ± 1.0; losartan 8.96 ± 0.96; $P = 0.74$) and $I_{ep}$ increased some 12% (pEC50 for control 9.79 ± 1.2 and for losartan 9.72 ± 1.1; $P = 0.97$). $R_{ep}$ was not influenced (Fig. 4).

Selective AT$_2$ receptor stimulation was performed by use of the nonpeptide AT$_2$ receptor agonist C21. A similar response pattern, although less pronounced, was obtained by C21 when given in two concentrations (0.1 and 1.0 μM) on the serosal side. Thus C21 increased significantly PD as well as $I_{ep}$ compared with the baseline ($P = 0.016$ and 0.028, respectively) but left $R_{ep}$ constant (Fig. 5).

Adding Ang II in presence of the selective AT$_2$ receptor antagonist PD123319 $10^{-6}$ M, serosal side) induced no significant effect on PD, whereas $I_{ep}$ decreased 10–12% ($P = 0.047$) and $R_{ep}$ increased by ~15% ($P = 0.01$) (Fig. 4).
During acidification the cationic luminal composition changes to a dominance of the H\(^+\) instead of Na\(^+\), adding a pronounced diffusion potential to the epithelial PD. The recorded magnitude of this diffusion potential is dependent on the electrical properties of the mucosa, i.e., \(I_{ep}\) and \(R_{ep}\) (5). It follows that it cannot be decided whether the observed changes in basal and acid-induced PD following candesartan intake were due to influencing \(I_{ep}\) or \(R_{ep}\) (or both). In any case, the fact that both basal PD and the acid-induced PD increment changed following candesartan strongly indicates involvement of endogenous Ang II in regulating epithelial functions. However, interferences with RAS in vivo are complicated also for pharmacological reasons. Although candesartan is a very selective AT\(_1\) receptor antagonist, it is not evident that effects observed following its administration are due to interference only with this Ang II subreceptor. Effects observed following administration of an AT\(_1\) receptor antagonist could also be due to actions by Ang II on the simultaneously unopposed AT\(_2\) receptor (7, 15, 28). The pharmacological background to this phenomenon deserves some explanation: Systemic administration of a selective AT\(_1\) receptor antagonist results in two important effects, the obvious blockade of the AT\(_1\) receptors and, as a result of the former, blockade of feedback inhibition of renin release resulting in increased renin activity. Being the main rate-limiting factor for Ang II formation, the increased renin activity results in an accelerated degradation of the circulating prohormone angiotensinogen to the decapeptide angiotensin I, which in turn will be degraded to the bioactive octapeptide Ang II by ACE (and other enzymes, e.g., chymase).

The AT\(_1\) receptor antagonist used in this study, candesartan, is a commonly prescribed antihypertensive and was given as a single dose that previously has been reported to increase renin release (2, 11). It is therefore reasonable to assume that endogenous Ang II formation increased markedly following the AT\(_1\) receptor antagonism and that the increased Ang II concentrations targeted mainly the AT\(_2\) receptor. However, it cannot be excluded that AT\(_1\) receptor interference raised transmucosal PD in the present study also by unloading AT\(_1\) receptors. Because there are no pharmaceuticals available for clinical use targeting the AT\(_2\) receptor, the evaluation of Ang II subreceptor involvement was performed in vitro in Ussing chambers. One advantage with experimentation on isolated mucosa in vitro is that concentration-response curves can be recorded from a baseline without background (endogenous) Ang II formation. In the present study we used conventional Ussing chambers demanding rather large esophageal specimens to provide stable experimental conditions. The mucosal tissue from the distal esophagus was obtained at surgical resections due to mucosal neoplasms. Great attention was paid to secure that the tissue under study was morphologically normal. It was also confirmed that gene and protein expressions of RAS components were present to the same degree and localization in resected tissues as observed in endoscopic biopsies from healthy volunteers.
Indeed, the in vitro study confirmed that Ang II influenced electrical parameters of the human esophageal mucosa via both the AT_1 and the AT_2 receptor subtypes. Administration of Ang II per se resulted in increased lumen negative PD due to an increased \( I_{ep} \) whereas \( R_{ep} \) was rather constant, indicating an increased lumen-directed net flux of anions or a serosally directed flux of cations. Because an identical response pattern was obtained also in presence of the AT_1 receptor antagonist losartan, activation of AT_1 receptors cannot be the dominant mechanism of action. Instead, the observed Ang II induced effects are to be ascribed to activation of AT_2 receptors. This interpretation was strongly supported by the finding that selective AT_2-receptor stimulation by the agonist C21 was also associated with an increased PD due to an increased \( I_{ep} \), without any apparent effect on \( R_{ep} \).

Interestingly, Ang II given in presence of the selective AT_2 antagonist PD123319 (thus a condition compatible with a selective AT_1 receptor stimulation) decreased \( I_{ep} \) and increased \( R_{ep} \), with the result that PD did not change significantly. These results are consistent with the existence of AT_1 receptor-mediated reductions of electrogenic ion transport and paracellular permeability.

The electrical negativity recorded in the esophageal lumen is mainly due to active transport of Na\(^+\) from lumen into the mucosa (1, 18, 25). It is therefore tempting to suggest that activation of esophageal epithelial AT_2 receptors increases Na\(^+\) absorption resulting in a more pronounced lumen negativity. In addition, bicarbonate secretion by the esophageal mucosa may constitute an alternative target for AT_2 receptor stimulation (26), as previously described for the rat duodenal mucosa (15, 29). Further studies are needed to confirm these possibilities and also to pinpoint which of several possible ion transport mechanisms (24) are involved. Although the present study indicates a physiological role for RAS in the esophageal mucosa, the degree of clinical significance with regard to, for example, epithelial resistance to acidification and other luminal aggressors related to gastroesophageal reflux remains to be investigated. In addition, in the renocardiocvascular system Ang II is ascribed an important role for promoting tissue proliferation (7). Recent epidemiological data show Ang II-induced contraction of rat and human small intestinal wall musculature in vitro. Acta Physiol (Oxf) 188: 33–40, 2006.


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