Endothelin Receptor Subtypes and Stimulation of Aldosterone Secretion

Celso E. Gomez-Sanchez, Eduardo N. Cozza, Mark F. Foecking, Shirley Chiou, and Mary W. Ferris

Endothelins (ETs) are 21-amino acid peptides with two disulfide bonds that have powerful vasoactive properties. We have previously shown the presence of a specific, high-affinity, saturable receptor for porcine or human endothelin (ET-1) in cultured calf zona glomerulosa cells. ET-1 was a stimulator of aldosterone secretion although not as powerful as angiotensin II. Incubations of cultured calf zona glomerulosa cells with Sarafotoxin S6b (S6b), a snake venom that has a structure highly homologous to ET-1, stimulated aldosterone secretion with a potency similar to that of ET-1. Binding of [¹²⁵I]ET-1 to the adrenal receptor gave a $K_d$ of $0.17\pm0.05$ nM and a $B_{max}$ of $36\pm8.5$ fmoles/well ($n=4$). Displacement of [¹²⁵I]ET-1 by unlabelled ETs and S6b showed that the concentrations needed to displace 50% of the tracer were 0.3 nM for ET-1, 0.3 nM for ET-2, 10 nM for S6b, and 100 nM for ET-3. Binding of [¹²⁵I]S6b to cultured adrenal cells revealed a receptor with a $K_d$ of $0.05\pm0.01$ nM and a $B_{max}$ of $8\pm2$ fmoles/well ($n=4$). Displacement of [¹²⁵I]S6b by unlabelled ETs and S6b showed that the concentrations needed to displace 50% of the tracer were 0.03 nM for S6b, 0.06 nM for ET-1, 0.04 nM for ET-2, and 0.05 nM for ET-3. Unlabeled ET-1 and ET-2 preferentially down-regulated the binding of [¹²⁵I]ET-1, and S6b preferentially down-regulated the binding of [¹²⁵I]S6b. Labeled S6b (which is unlikely to exist in mammals) served as a tool to uncover the high-affinity receptor for ET-1 (ET-1a receptor), which is likely responsible for stimulation of aldosterone secretion. The second receptor or lower affinity, higher capacity receptor (ET-1β receptor) has an unclear function at this time. (Hypertension 1990;15:744–747)
Endothelin Receptor Subtypes

International (Louisville, Kentucky). Protein concentration was measured using the BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, Illinois).

Iodination of Endothelin-1, Endothelin-3, and Sarafotoxin S6b

The iodination with $^{125}$I and high-performance liquid chromatography purification was done as previously described.

Culture of Adrenal Zona Glomerulosa Cells

Calf adrenal zona glomerulosa cells were cultured in six- or 24-well plates as previously described. The protein concentration was 26.6±0.4 μg/well (mean±SEM, n=32). Cells were plated at a concentration of 200,000 cells/well, and at confluence, there were 438,000±8,300 cells/well (mean±SEM, n=12).

Binding of $[^{125}]$Endothelin-1, $[^{125}]$Endothelin-3, and $[^{125}]$Sarafotoxin to Adrenal Cells

The binding of the iodinated peptides to calf zona glomerulosa cells was done as previously described.

Incubation of Adrenal Zona Glomerulosa Cells With Endothelins and Sarafotoxin

Cells grown for 3–4 days in 24-well plates were incubated with increasing concentrations of ETs and S6b for 2 hours at 37° C in a 5% CO₂ atmosphere. Control incubations with $10^{-9}$ M Ang II were also done. Aldosterone secretion was measured by direct radioimmunoassay with a monoclonal antibody. Statistical analysis of the differences between groups was done by analysis of variance using STATVIEW 512+ Program (Brain Power, Calabazas, California) for the Macintosh computer.

Down-regulation of Endothelin-1 and Sarafotoxin S6b Receptors

Cells were incubated for 2 hours with 1 nM peptide, washed three times with Hanks’ balanced salt solution, incubated 2 hours with Ham F-12 containing 0.2% bovine serum albumin, and washed, and then receptors were measured as previously described. $B_{max}$ was measured using a Scatchard plot. To be certain that these washing conditions eliminated all bound peptide before measuring receptor number, cells were incubated under identical conditions with $[^{125}]$ET-1, and the amount of radioactivity was measured after washing. Less than 5% of bound counts remained after the washing procedure.

Results

The binding kinetics of $[^{125}]$ET-1 have been previously published, and an experiment is shown with the plot for $[^{125}]$S6b. Scatchard analysis revealed a single site with an apparent dissociation constant ($K_d$) of 0.17±0.05 nM and a concentration of binding sites of 36±8.5 fmol/well (n=4). Binding of $[^{125}]$S6b was time dependent and reached an apparent equilibrium at 60 minutes. Nonspecific binding was less than 10% of bound radioactivity. Binding of S6b was saturable, and Scatchard analysis revealed the presence of a single class of high-affinity binding sites with an apparent $K_d$ of 0.05±0.01 nM with a concentration of binding sites of 8±2 fmol/well (n=4) (Figure 1). Binding obtained with $[^{125}]$ET-3 was much lower (about 15% of that of ET-1 or S6b), and for this reason Scatchard plots for ET-3 binding were not usable for calculation of binding parameters.

Displacement of $[^{125}]$ET-1 by unlabeled ET and S6b showed that the concentrations needed to displace 50% of the tracer were 0.3 nM for ET-1, 0.3 nM for ET-2, 10 nM for S6b, and 100 nM for ET-3 (Figure 2). Displacement of $[^{125}]$S6b by unlabeled ET and S6b showed that the concentrations needed to displace 50% of the tracer were 0.03 nM for S6b, 0.06 nM for ET-1, 0.04 nM for ET-2, and 0.05 nM for ET-3 (Figure 3).

ET-1 produces down-regulation of its receptors. When cells were treated with unlabeled ET-1 and were extensively washed, measurement of $[^{125}]$ET-1 binding and $[^{125}]$S6b binding showed that the $B_{max}$ for ET-1 was decreased by 50% and for S6b was decreased by 25% (Table 1). Pretreatment with ET-2 instead of ET-1 provoked down-regulation of ET-1.
and S6b receptors to the same extent (Table 1). This finding is in agreement with the displacement studies that suggested that ET-1 and ET-2 bind to the same site or sites. In cells pretreated with ET-3, the ET-1 receptors were not affected nor surprisingly were the S6b receptors because ET-3 displaced S6b so well. Pretreatment with unlabeled S6b decreased the ET-1 binding by 28% and S6b binding by 40%. These results are in agreement with the hypothesis of one shared receptor for ET-1 and S6b, which is down-regulated by both, and another receptor for ET-1, which is not affected by S6b pretreatment but down-regulated by ET-1 and to which S6b preferentially binds.

**Effect of Endothelin-1 and Sarafotoxin S6b on Aldosterone Production**

ET-1 and S6b stimulated aldosterone secretion to a similar degree (Figure 4).

### Discussion

We have previously shown and further confirmed that the ETs bind to adrenal receptors and are capable of stimulating aldosterone secretion but with a significant lesser potency than Ang II.8 The highly homologous snake venom S6b was also capable of stimulating aldosterone secretion with a potency similar to ET-1. ET-1 and S6b were equal in their ability to stimulate aldosterone secretion; however, S6b displaced [125I]ET-1 from the receptor with only 3% of the potency of ET-1. This discrepancy suggested the possibility of a second adrenal receptor for these peptides. Binding studies with [125I]S6b revealed the presence of a second receptor that had a higher affinity for all the ETs and S6b tested than the first ET-1 receptor revealed by using [125I]ET-1 tracer. The [125I]S6b also demonstrated a hierarchy of displacement that was very similar for all the ETs tested. This suggests that the stimulation of aldosterone secretion occurs most likely through a second receptor as revealed by [125I]S6b binding. There is no evidence to indicate that S6b exists in mammals; however, its strong structural homology makes it a useful investigative tool. These studies suggest that there are two ET-1 receptors, but because of the high affinity of ET-1 for both receptors, the Scatchard plot using [125I]ET-1 studies was unable to give enough resolution to suggest the presence of a second receptor. The high-affinity receptor (Bmax of 8 fmol/well and Kd of 0.05 nM) we call the ET-1α receptor, and the previously described receptor (Bmax of 36 fmol/well [actually 36-8 fmol/well]) we are calling the ET-1β. S6b served as a tool to uncover the existence of the first receptor.

Further evidence that these are two different receptors is that down-regulation of the receptors is different according to which peptide is used for its induction. S6b preferentially down-regulates the higher affinity, lower capacity, ET-1α receptor, and ET-1 or ET-2 best down-regulates the previously described ET-1β receptor.8 It is interesting that ET-3 competes very poorly with [125I]ET-1 but very effectively against [125I]S6b for binding. ET-3 is a very poor stimulator of aldosterone secretion.8 This pattern of

### Table 1. Receptor Down-Regulation

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[125I]ET-1</th>
<th>[125I]S6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±4.2*</td>
<td>100±3.8†</td>
</tr>
<tr>
<td>ET-1 (nM)</td>
<td>48±2.6*</td>
<td>75±0.9‡</td>
</tr>
<tr>
<td>ET-2 (nM)</td>
<td>51±3.1‡</td>
<td>73±4.7‡</td>
</tr>
<tr>
<td>ET-3 (nM)</td>
<td>102±6.9†</td>
<td>99±8.1†</td>
</tr>
<tr>
<td>S6b (1 nM)</td>
<td>73±2.3†</td>
<td>54±3.6‡</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Bmax, maximal binding; [125I]ET-1, [125I]endothelin-1; [125I]S6b, [125I]Sarafotoxin S6b; ET-1, 2, 3, endothelin-1, 2, 3, respectively; S6b, Sarafotoxin S6b.

* n=5, † n=4, ‡ n=3, number of experiments.
ET-1 receptor because ET-3 is slightly more potent than ET-1 for binding to this receptor. It is very likely that the binding of ET-3 to the S6b-prefering receptor results in the formation of a peptide-receptor complex that does not activate the mediators of stimulation. Further evidence for this hypothesis is provided by the inability of ET-3 to induce down-regulation of either receptor (Table 1). There is the possibility (that was not tested) that ET-3 might have properties antagonist to the other ETs.

Receptor heterogeneity for ETs has been described for other tissues. Chick cardiac membranes exhibit two types of receptors, one ET-1,2-preferring receptor that exhibits an order of potency of ET-2≥ET-1>ET-3>S6b and a second type, or ET-3-preferring receptor, that exhibits an order of potency of ET-3>ET-2>ET-1≥S6b. In the order of S6b potency, these receptors are different from those described in the adrenal gland. Kloo et al described three apparent receptor subtypes, that is, a high-affinity ET-1/S6b subtype of smooth muscle, a high-affinity S6c subtype typical of the cerebellum, and a less selective subtype typical of the caudate putamen that binds all these peptides with high affinity. It is apparent that there is a great heterogeneity in receptor subtypes; however, the significance is not clear because only ET-1 has been isolated from tissue. The structures of other ETs have been determined by using the nucleotide sequence of cDNAs isolated with a probe against ET-1, 4-6 and it is very likely that the binding of ET-3 to the S6b-preferring receptor subtypes, that is, a high-affinity ET-1/S6b subtype of smooth muscle, a high-affinity S6c subtype typical of the cerebellum, and a less selective subtype typical of the caudate putamen that binds all these peptides with high affinity. It is apparent that there is a great heterogeneity in receptor subtypes; however, the significance is not clear because only ET-1 has been isolated from tissue. The structures of other ETs have been determined by using the nucleotide sequence of cDNAs isolated with a probe against ET-1, 4-6 and it is not known if and where gene expression for these peptides occurs.

ET-1 action in the adrenal is likely to be caused by an increased secretion at a paracrine site because circulating amounts of ET-1 are very small. Malignant hypertension is associated with an increased secretion of aldosterone in a significant number of cases. On treatment, the aldosterone secretion normalizes much later than the renin-angiotensin system, the main stimulator of aldosterone secretion, suggesting the action of an additional aldosterone stimulator or stimulators, one of which might be ET-1. Significant endothelial changes and damage occur in malignant hypertension, which might lead to increased secretion of ET-1. The role of ET-1 in the acceleration of the disease process in malignant hypertension is speculative at this time, but if ET-1 does have a role, then aldosterone overproduction induced by ET-1 is a reasonable hypothesis.

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References
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