Autocrine Role for the Endothelin-B Receptor in the Secretion of Adrenomedullin

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Abstract—Adrenomedullin, originally discovered in human pheochromocytoma, is a vasodilating and natriuretic peptide of vascular endothelial and smooth muscle cell origin. Although endothelin-1 (ET-1) has been implicated as a vasoconstricting and growth-promoting peptide of endothelial origin, it may more importantly function as an autocrine factor and release vasodilatory substances such as nitric oxide by mechanisms linked to the endothelin-B (ET_B) receptor subtype. The present study was designed to establish that the ET_B receptor stimulates the secretion of adrenomedullin from cultured canine aortic endothelial cells. We first sought to determine the presence and production of adrenomedullin in canine aortic endothelial cells using immunohistochemistry and Northern blot analysis, which revealed that adrenomedullin immunoreactivity and adrenomedullin mRNA were present in canine aortic endothelial cells. Second, adrenomedullin was time-dependently secreted from canine aortic endothelial cells, with a secretion rate of 15.7±1.5 pg/10^5 cells per 24 hours. Furthermore, immunohistochemistry revealed the presence of the ET_B receptor in canine aortic endothelial cells, and ET_B receptor stimulation by sarafotoxin S6c increased adrenomedullin production and secretion from canine aortic endothelial cells. Such actions were blocked with the ET_B receptor antagonist IRL-2500 but not with ET_A receptor antagonist FR-139317. These studies are the first to report an additional autocrine role of the ET_B receptor in the release of vasodilating and natriuretic peptide adrenomedullin, and they suggest another important vasoactive system regulated by the ET receptor subtype. (Hypertension. 1998;32:917-922.)

Key Words: endothelium ■ hormones ■ immunohistochemistry ■ peptides ■ radioimmunoassay

Adrenomedullin (ADM) is a 52-amino acid vasodilating and natriuretic peptide that possesses an intramolecular disulfide bond and C-terminal amide structure. Its biological actions involve activation of the second messenger cAMP. The presence of specific ADM receptors on vascular smooth muscle cells, along with studies that have reported the synthesis and secretion of ADM in vascular endothelial cells and smooth muscle cells, supports the concept that ADM may play an important autocrine and/or paracrine role in the regulation of vascular tone. Furthermore, ADM has been detected in normal human plasma, and its circulating concentrations are reported to be increased in patients with hypertension, chronic renal failure, and congestive heart failure. To date, however, control of ADM secretion has not been well characterized.

The vasoconstricting and mitogenic peptide endothelin-1 (ET-1) is released from vascular endothelial cells under normal and pathophysiological conditions and plays an important autocrine and/or paracrine role in the regulation of vascular tone. ET-1 exerts its biological actions through at least 2 receptor subtypes, the endothelin-A (ET_A) and endothelin-B (ET_B) receptors. While the ET_A receptor may mediate vasoconstriction under pathophysiological conditions, the ET_B receptor, which is localized to the endothelial and smooth muscle cells, may function under physiological conditions to mediate vasodilation via release of potent vasodilators that include nitric oxide (NO) and prostacyclin (PGI_2). Although both ET-1 and ADM are secreted from the vascular endothelial cells, little is known about the relationship between ET-1 and ADM. In vitro studies by Sugo et al have demonstrated that ET-1 stimulates ADM secretion from cultured vascular smooth muscle cells. To date, however, no in vitro studies have investigated the role of the ET_B receptor in the release of ADM in vascular endothelial cells. We hypothesized that activation of the ET_B receptor increases ADM secretion from vascular endothelial cells. To test this hypothesis, we investigated the presence and production of ADM in cultured canine aortic endothelial cells (CAECs) and defined the role of the ET_B receptor by determining its presence in CAECs and by characterizing ADM production and secretion in response to the ET_B receptor agonist sarafotoxin S6c. Last, we assessed whether ET_B receptor blockade would attenuate such actions.

Methods

Cell Culture

CAECs were prepared in a similar fashion as described previously. Briefly, endothelial cells were isolated from male mongrel dogs by
scraping the intimal surface of the aorta with a scalpel and were cloned and maintained in Medium 199 (Bionhwittaker) supplemented with 10% fetal calf serum at 37°C and 5% CO₂ with 95% O₂ in a humidified atmosphere. CAECs were identified by active production of ET-1, positive immunostaining with monoclonal anti-von Willebrand factor antibody, and negative immunostaining for α-smooth muscle cell actin. At confluence, CAECs appeared as typical “cobblestone”-patterned monolayers. CAECs in the fourth to eighth passage were used in the present experiments.

**Radioimmunoassay for ADM**

After incubation for the indicated time, the culture medium was removed and immediately acidified with acetic acid (final concentration, 1 mol/L), heated at 100°C for 10 minutes in 20°C until assay. Culture medium (1 mL) was extracted on C-18 Bond Elute cartridges and eluted with 75% methanol containing 1% trifluoroacetic acid. Concentrated eluates were then assayed using a specific and sensitive radioimmunoassay for ADM(1-52) (Phoenix Pharmaceuticals Inc) as previously described. Minimal detectable concentration for the assay is 1 pg per tube, and the half-maximal inhibition dose of radiiodinated ligand binding by ADM is 20 pg per tube. Recovery is 72%, and intra-assay and interassay variations are 10% and 12%, respectively.

**Radioimmunoassay for ET-1**

The measurement of ET-1 in the culture medium was performed using a specific and sensitive radioimmunoassay for ET-1 as previously reported. Culture medium (1 mL) was extracted on C-8 Bond Elute cartridges and eluted with 95% methanol containing 1% trifluoroacetic acid. Concentrated eluates were then assayed. Minimal detectable concentration for the assay is 0.5 pg per tube, and the half-maximal inhibition dose of radiiodinated ligand binding by ET-1 is 10 pg per tube. Recovery is 71%, and intra-assay and interassay variations are 9% and 5%, respectively.

**Isolation of mRNA**

Isolation of mRNA from canine adrenal glands was performed using the Micro-FastTrack kit (Invitrogen) following the manufacturer’s instructions. In brief, the adrenal glands were initially lysed in detergent-based buffer containing RNase/Protein Degrader, incubated at 45°C, and applied directly to oligo(dT) cellulose for adsorption. DNA, degraded proteins, and cell debris were washed from the resin with a high-salt buffer. Nonpolyadenylated RNA was washed off with a low-salt buffer, and the poly(A) RNA was then eluted in the absence of salt. Purification and quality of the RNA was assessed by reading optical densities at 260 and 280 nm and by electrophoresis in 1.2% agarose gel.

**Reverse Transcription-Polymerase Chain Reaction**

The cDNA sequence for canine ADM has not been reported. To obtain a specific probe for canine ADM, reverse transcription–polymerase chain reaction (RT-PCR) was performed using primers that are highly conserved among species. The cDNA was prepared from 1 μg mRNA using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer). The oligonucleotide primers were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer (Perkin-Elmer). After transcription, the following porcine primers were selected from the published sequence:

- sense (porcine ADM 91-113): 5'-CGAAGAGAACGAGTGGATGTTGAC-3';
- antisense (porcine ADM 364-383): 5'-GTGAACTGCTGATCCTGTGG-3', yielding a predicted product of 293 bp. A Perkin-Elmer 2400 Thermocycler was used to amplify the samples. PCR cycling parameters were as follows: initial denaturation at 94°C for 3 minutes followed by cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Thirty-five cycles were used with a final extension time of 7 minutes. The PCR products were cloned into a TA cloning vector (Invitrogen) and sequenced by ABI Prism dyeoxy chain termination method using Applied Biosystems 377 Automated DNA Sequencer. The nucleotide sequence is reported in Figure 1. The clone contained a 293-bp fragment, which was considered to encode the deduced structure of canine ADM (GenBank accession number AF045773).

**Northern Blot Analysis**

For Northern blot analysis, 4 μg mRNA from the cell extracts was loaded on a 1.2% agarose formaldehyde gel and electrophoresed for 3 hours at 70 V. The gel was transferred downward (Turboblotter, Schleicher & Schuell) onto a nylon membrane (Maximum Strength Nytran Membrane, Schleicher & Schuell) overnight. An EcoRI DNA...
A restriction fragment containing 293 bp gene for canine ADM was labeled with $^{32}$P-dCTP by a random-priming labeling kit (Megaprime DNA labeling system, Amersham) and purified using G-50 NICK Spin Columns (Pharmacia Biotech). The membranes were then washed in $2\times$ SSC, 0.1% SDS at 22°C for 15 minutes, then in $0.2\times$ SSC, 0.1% SDS at 22°C for 15 minutes, and $0.2\times$ SSC, 0.1% SDS at 55°C for 20 minutes. Autoradiography was carried out with Kodak x-ray film at $-80°C$ overnight. To control for loading conditions and mRNA transfer onto membranes, blots were rehybridized with a GAPDH probe.

**Statistical Analysis**

Results of quantitative studies are expressed as mean±SEM. Statistical comparisons were performed using ANOVA for repeated measures followed by Fisher’s least significant difference test when appropriate. Comparisons between groups were performed using Student’s unpaired $t$ test. Statistical significance was accepted at a value of $P<0.05$.

**Results**

**Immunohistochemical Stainings for ADM and ET<sub>B</sub> Receptor in CAECs**

Positive immunostaining for ADM was observed in the cultured CAECs. Representative immunohistochemical staining for ADM in CAECs is illustrated in Figure 2. ADM immunoreactivity was observed within the cells and distributed widely in the peripheral cytoplasm. There was also ADM immunoreactivity in the perinuclear regions. The cells treated with preabsorbed antiserum, nonimmune rabbit serum, or PBS instead of primary antibody demonstrated little or no ADM immunoreactivity.

Positive immunostaining for the ET<sub>B</sub> receptor was also detected in the cultured CAECs. Representative immunohistochemical staining for the ET<sub>B</sub> receptor in CAECs is illustrated in Figure 3. The distribution of ET<sub>B</sub> receptor immunoreactivity was similar to that of ADM immunoreactivity. The cells treated with nonimmune horse serum or PBS instead of primary antibody demonstrated no immunoreactivity.

**Basal and Stimulated ADM Secretion From CAECs**

CAECs spontaneously secreted ADM into the culture medium. Figure 4 illustrates the time course of ADM secretion in the presence or absence of sarafotoxin S6c ($10^{-7}$ mol/L) into the culture medium. ADM concentration in culture medium from the CAECs increased in a time-dependent manner up to 24 hours. The spontaneous secretion rate was $15.7\pm1.5$ pg/10<sup>5</sup> cells per 24 hours. The ET<sub>B</sub> receptor stimulation by sarafotoxin S6c significantly increased ADM secretion ($21.2\pm1.5$ pg/10<sup>5</sup> cells per 24 hours, $P<0.05$ versus control secretion). Figure 5 illustrates the dose-response effect of sarafotoxin S6c on the secretion of ADM. Incubation of the CAECs with sarafotoxin S6c ($10^{-8}$ to $10^{-7}$ mol/L) for 16 hours increased ADM secretion from the CAECs. Figure 6 illustrates the effects of the ET<sub>A</sub> receptor antagonist FR-139317 ($10^{-6}$ mol/L) and ET<sub>B</sub> receptor antagonist IRL-2500 ($10^{-6}$ mol/L) on the secretion of ADM in the presence and absence of sarafotoxin S6c ($10^{-9}$ to $10^{-7}$ mol/L) for 16 hours. The ET<sub>B</sub> but not ET<sub>A</sub> receptor antagonist blocked stimulated secretion of ADM by sarafotoxin S6c. IRL-2500 alone decreased ADM secretion, suggesting that basal ET-1 secretion regulates ADM secretion via the ET<sub>B</sub> receptor. The decreased ADM secretion by IRL-2500 was reversed by adding excess sarafotoxin S6c ($10^{-7}$ mol/L). Viability of CAECs after 24 hours of incubation was >98% as determined by trypan blue staining.

**ET-1 Secretion From CAECs**

To determine endogenous ET-1 secretion, which could stimulate ADM secretion, we measured ET-1 in the culture medium. CAECs spontaneously secreted ET-1 into the culture medium. Figure 7 (top) illustrates the time course of ET-1 secretion into the culture medium. ET-1 concentration in culture medium from the CAECs increased in a time-dependent manner up to 24 hours. The spontaneous secretion rate of ET-1 was $188.3\pm31.1$ pg/10<sup>5</sup> cells per 24 hours, which is 13 times higher than ADM secretion rate. Figure 7 (lower...
Northern Blot Analysis
Northern blot analysis of ADM mRNA from the cultured CAECs in the presence and absence of sarafotoxin S6c is illustrated in Figure 8. Northern blot analysis demonstrated the presence of ADM mRNA in the cultured CAECs that was \( \approx 1.6 \) kb in size, indicating the production of ADM in the CAECs. The ET\(_B\) receptor stimulation with sarafotoxin S6c (\(10^{-7}\) mol/L) significantly increased ADM gene expression in the cultured CAECs (Figure 8).

Discussion
The objective of the present investigation was to confirm the endothelial cell as a site of presence, production, and secretion of ADM and to define the role of the ET\(_B\) receptor in the synthesis and release of this peptide. The present findings establish the presence of ADM immunoreactivity in the endothelial cells by immunohistochemistry, ADM production in the endothelial cells by Northern blot analysis, and ADM secretion from the endothelial cells by radioimmunoassay. We have also demonstrated that ADM production and secretion are increased by activation of the ET\(_B\) receptor in endothelial cells. Furthermore, this enhanced secretory response was blocked by an ET\(_B\) receptor antagonist. Thus, the present investigation supports an important autocrine interaction between the ADM and ET systems in the control of ADM secretion.

ADM has emerged as a vasoactive peptide originally isolated from pheochromocytoma cells.\(^1\) Its biological properties include vasodilatation and natriuresis, with conflicting data related to growth regulation\(^{18,19}\) and inotropism.\(^{20,21}\) While its physiological role continues to emerge, studies suggest its activation in states of cardiorenal dysfunction such as hypertension\(^6\) and congestive heart failure,\(^7,8\) where it may play a compensatory role to limit excessive vasoconstriction.

The present investigation confirms the important concept that ADM is an endothelial cell–derived vasoactive peptide in addition to being secreted by vascular smooth muscle cells. Specifically, the present study, using both immunohistochemical staining of CAECs and radioimmunoassay of cell culture media, establishes the presence and secretion of ADM from the endothelial cell. Thus, ADM emerges as another endothelial cell–derived vasodilatory factor complementing NO, PGI\(_2\), and C-type natriuretic peptide.

Sugo et al\(^{14}\) have reported factors that augment ADM secretion from cultured vascular smooth muscle cells. One such factor is ET-1. The present study importantly extends our understanding of an interaction between ET-1 and ADM by demonstrating that stimulation of the ET\(_B\) receptor with sarafotoxin S6c increases the production and secretion of ADM.
ADM from the endothelial cells. The ability of an ETB receptor antagonist, IRL-2500, to attenuate the action of sarafotoxin S6c further establishes that ADM secretion is linked to the ETB receptor. Moreover, ETA receptor antagonism with FR-139317 failed to attenuate the action of the ETB agonist. Of interest is the additional observation that IRL-2500 alone significantly decreased ADM secretion, suggesting that via an autocrine action, basal secretion of ET-1 from the endothelial cells regulates ADM secretion via the ETB receptor.

The endocrine role of the ETB receptor has been intensively investigated and indeed may represent the primary role in mediation of the actions of ET-1 under physiological conditions. To date, studies support the ability of the ETB receptor to mediate endothelial release of vasodilatory factors that include NO and PGI2.12,13 The present study extends this endocrine role to ADM. Indeed, these observations may provide some understanding of the continuing vasorelaxing action of ETB stimulation in isolated vessels with endothelium intact when the NO and PGI2 systems are inhibited. Furthermore, it is tempting to speculate that if the endothelium is exposed to physiological stimuli that release ET-1 at very low concentrations, the primary action of the ET system may be in the autocrine release of vasodilatory factors, underscoring a primary vasodilatory rather than constrictor role for ET-1.

Sugo et al5,14 have reported that cultured vascular smooth muscle cells secrete a considerable amount of ADM into the culture medium. Although we did not demonstrate ETB-mediated ADM secretion from the canine vascular smooth muscle cells, ADM is thought to be secreted via ETB receptors, which are also localized to vascular smooth muscle cells.

Recently, cDNA clones encoding human,22 porcine,17 mouse,21 and rat ADM precursors were isolated and sequenced. These reports indicate that the structure of mammalian ADM is well conserved among species, and thereby raise the possibility that antiserum to human ADM could also detect ADM of other mammalian species, such as dogs. In the present study, we used polyclonal anti-human ADM antiserum to detect canine ADM in the CAECs. Although canine ADM has not been isolated, the positive immunostaining of the present study are considered to be “canine ADM.” With this polyclonal antibody, the present study demonstrates the presence of ADM in CAECs, which may be consistent with the endothelial cells being important sites for ADM production. Northern blot analysis supported this ADM production in CAECs. As for the ETB receptor, although the canine ETB receptor has not been determined, the positive staining for ETB receptor is considered to be “canine ETB receptor” as explained similarly above.

The present study in cultured CAECs may have pathophysiological relevance. To date, in studies of congestive heart failure in which circulating ET-1 is increased, ADM has also...
been reported to be increased.\textsuperscript{7,8} It is tempting to speculate that in a state of excessive ET-1 release, as in congestive heart failure, ET\textsubscript{B} receptor stimulation may explain in part the increase in circulating and tissue ADM\textsuperscript{14,25} reported in such disease states.

In summary, the present study advances our understanding of the vascular biology of ADM, specifically as an endothelial cell–derived vasodilatory peptide. In addition, its secretion from vascular endothelial cells is enhanced by the ET\textsubscript{B} receptor activation and decreased with ET\textsubscript{A} receptor blockade. Thus, these studies are the first to establish an autocrine role for the ET\textsubscript{B} receptor in the release of the vasodilating peptide ADM and suggest another important vasoactive system regulated by the ET\textsubscript{B} receptor.

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References
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