Modulation of Endothelin Effects on Blood Pressure and Hematocrit by Atrial Natriuretic Peptide

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Infusion of endothelin has been observed to increase hematocrit, and the peptide also stimulates release of atrial natriuretic peptide (ANP) both in vitro and in vivo. We studied the relation of these two actions of endothelin in anesthetized, bilaterally nephrectomized Sprague-Dawley rats. Infusion of endothelin (25 ng/kg/min) for 45 minutes produced a modest increase in blood pressure of 12% from a baseline of 99±5 mm Hg and an increase in hematocrit of 8.0±0.6%, reflecting a reduction in plasma volume of 13.1±0.9%. These changes each exceeded greatly those observed after 45 minutes of vehicle infusion. Plasma protein concentration, however, increased only by 4.2±0.6%, suggesting protein extravasation, which was confirmed by finding an endothelin-dependent increase in the accumulation of Evans blue dye in heart, skeletal muscle, and intestine, but not liver, lung, brain, or testis. Endothelin infusion increased plasma immunoreactive ANP concentration from 196±50 to 722±203 pg/ml (p<0.02), and a close correlation existed between the increase in plasma immunoreactive ANP and immunoreactive endothelin concentrations as a result of the infusion (r=0.84, p<0.01). Pretreatment of rats with rabbit anti-rat ANP antiserum did not affect baseline variables but led to an exaggerated increase in blood pressure (25.3±2.9%, p<0.002 versus endothelin alone). No change in hematocrit occurred. Thus, the increase in plasma immunoreactive ANP concentration resulting from endothelin infusion mediates the increase in hematocrit through an increase in vascular permeability to whole plasma. The exaggerated pressor effect of the infused endothelin in the presence of ANP antiserum suggests that ANP may modulate the vasoconstrictor actions of endothelin in vivo. (Hypertension 1991;17:864-869)

The recently characterized peptide endothelin has numerous actions relating to cardiovascular homeostasis.1-3 In addition to its potent vasoconstrictor properties, it influences renal function and affects several hormonal systems participating in body fluid regulation.2-5 Among these is a stimulation of atrial natriuretic peptide (ANP) release both in vivo and in vitro.4-8 Endothelin also causes an increase in hematocrit not fully accounted for by urinary fluid loss, suggesting that this hemoconcentration could result from a vascular leakage.4,5,10 Because ANP also causes hemoconcentration, through a mechanism involving an increase in vascular permeability,11-14 we carried out experiments to examine whether the increase in hematocrit caused by infusion of endothelin reflected an increase in vascular permeability in turn resulting from an endothelin-stimulated increase in plasma ANP concentration. Our results indicate that this is indeed the case.

Methods

We carried out studies in male Sprague-Dawley rats (Bantin and Kingman, Inc., Fremont, Calif.) weighing 250–360 g and allowed them free access to food and water until the day of experiment. Animals were anesthetized with an intraperitoneal injection of 100 mg/kg Inactin (Byk-Gulden, Konstanz, FRG) and placed on a heated table to maintain rectal temperature at 37±0.5°C. Animals underwent tracheostomy and breathed spontaneously; they were prepared for acute experimentation as previously described.13 Briefly, catheters were inserted into a femoral artery and vein, the right carotid artery, and the right jugular vein for sampling of blood, infusion of fluids and drugs, and continuous measurement of
arterial and venous pressures with a Statham P23 ID pressure transducer (Gould Instruments, Oxnard, Calif.) connected to a polygraph (model 7D, Grass Instrument Co., Quincy, Mass.); the jugular venous catheter was positioned at the level of the right atrium. Both kidneys then were removed through bilateral flank incisions. During surgical preparation, rats received a constant intravenous infusion of plasma substitute (Hespan, 6% hetastarch in 0.9% sodium chloride, Du Pont Pharmaceuticals, Wilmington, Del.) at a rate of 40 \mu l/min via a syringe pump (Harvard Apparatus, South Natick, Mass.), until a total volume of 0.5% body weight was administered to replace estimated fluid losses. Thereafter, the infusion rate was reduced to 10 \mu l/min for the duration of the studies. Experiments were started 30–45 minutes after completion of surgical procedures.

**Effect of Endothelin on Arterial Pressure, Hematocrit, and Plasma Protein Concentration**

After a 45-minute control period, rats received either rat endothelin-3 (Peninsula Laboratories, Inc., Belmont, Calif.) at a dose of 25 ng/kg/min (n=9) or the vehicle (0.9% sodium chloride containing 1 mg/ml bovine serum albumin and 1 mg/ml bacitracin, n=10). In addition, endothelin also was infused in four binephrectomized rats that had undergone splenectomy during surgical preparation. The infusion of endothelin or vehicle was at a rate of 10 \mu l/min for 45 minutes. At the end of the experimental period, a 45-minute period was allowed for recovery. Three 50-\mu l blood samples were taken at 15, 30, and 45 minutes during the basal, experimental, and recovery periods, respectively, for determination of hematocrit and plasma protein concentration (PPC). Packed cells were suspended in saline and returned to the animal. Hematocrit was measured in duplicate by refractometry (National Adams, Parsippany, NJ.) for 3 minutes. PPC was estimated by spinning blood at 12,000 rpm in a microfuge (Clay Instrument Co., Inc., Baltimore, Md.).

**Effect of Endothelin on Extravasation and Tissue Accumulation of Plasma Albumin**

These experiments were conducted in two groups of 10 binephrectomized rats each receiving either the vehicle or 25 ng/kg/min endothelin to estimate the extravasation of albumin using Evans blue dye.\(^{13,15,16}\) After surgical preparation and stabilization of arterial pressure, a 5 mg/kg dose of Evans blue diluted in 0.9% saline (5 mg/ml) was injected intravenously. A blood sample was obtained 2 minutes later for the determination of hematocrit. Five minutes after Evans blue injection, infusion of the vehicle or endothelin began. After a 30-minute period of infusion, blood samples were obtained for the determination of hematocrit, and the rats were killed. Intravascular fluid was washed out rapidly with plasma substitute injected through the carotid artery and drained through the vena cava. Several organs (brain, liver, heart, mesentery, lung, spleen, small intestine, skeletal muscle, and testis) were removed and briefly rinsed in isotonic saline. Samples then were blotted dry, weighed, and left at room temperature for 4–5 days in 4 ml formamide (Sigma Chemical Co., St. Louis, Mo.). The extracted amount of Evans blue was measured in a spectrophotometer at 620 nm (Gilford Instruments, Medfield, Mass.) and expressed as micrograms per gram of wet tissue.

**Effect of Endothelin on Plasma Concentration of Atrial Natriuretic Peptide**

After surgical preparation and stabilization of arterial pressure, 2.5 ml arterial blood was rapidly transferred into an ice-cold tube containing the following protease inhibitors: 1 mg EDTA, 1.2 trypsin inhibitor unit aprotinin, 10 \mu g pepstatin A, and 100 \mu g phenylmethylsulfonyl fluoride (Sigma) per milliliter blood. Blood was immediately centrifuged (4,000 rpm) at 4°C for 15 minutes, and plasma was kept frozen at -70°C until subsequently extracted and analyzed for immunoreactive ANP and endothelin. The blood was replaced by an equal volume of whole blood freshly harvested from a donor rat. After a 45-minute control period, endothelin (n=13) was infused for 45 minutes. A second arterial blood sample was obtained at the end of the experimental period, and the animals were killed.

**Effect of Rabbit Anti-Rat Atrial Natriuretic Peptide Antiserum on the Response to Endothelin**

To test the importance of changes in circulating immunoreactive ANP on the response to exogenously administered endothelin, endothelin (25 ng/kg/min) was infused in rats given rabbit ANP antiserum (n=10) or nonimmune rabbit serum (n=4). The ANP antiserum was raised in a New Zealand White rabbit immunized with a conjugate of rat ANP-(5–27) (Peninsula Laboratories) and thyroglobulin (Sigma). The antiserum shows complete cross-reactivity with rat and human ANP-(1–27). A volume of 70 \mu l of the control serum or antiserum was injected in the middle of the control period, at the beginning of the experimental period, and again at the beginning of the recovery period. The efficacy of the antiserum was assessed in a separate group of six animals by measuring the diuretic, natriuretic, and kaliuretic response to a bolus injection of 1 \mu g rat ANP (Peninsula Laboratories) before and after administration of the antiserum (70 \mu l).

**Analytical Techniques, Calculations, and Statistical Evaluation**

Plasma concentration of immunoreactive ANP was determined by radioimmunoassay after extraction on a Sep-Pak C\(_{18}\) column (Waters Chromatography Division, Millipore Corp., Milford, Mass.) preequilibrated with 0.1% trifluoroacetic acid as previously described.\(^{17}\) After elution with 75% methanol in 0.1% trifluoroacetic acid and evaporation to dryness under a stream of nitrogen, the residue was reconstituted in assay buffer and measured for ANP concentration using a radioimmunoassay kit (Peninsula Laboratories) that is specific for rat ANP. The antiserum shows complete cross-reactivity with rat and human ANP-(5–27).
immunoreactivity with a commercially available antiserum (Research and Diagnostic, Emeryville, Calif.) and \(^{125}\)I-ANP-(1-28). The sensitivity of this assay was 5 pg per tube. Intra-assay and interassay coefficients of variation were 5% and 16%, respectively.

Plasma concentration of endothelin was determined by radioimmunoassay after extraction of plasma samples on a Sep-Pak column pretreated with successive application of 4 ml 0.1% trifluoroacetic acid and 20 ml 60% acetonitrile. After sample application, these solvents were reapplied. After evaporation to dryness in a centrifugal concentrator (Savant Instruments, Inc., Farmington, N.Y.) and reconstitution of the residue in 500 \(\mu\)l assay buffer, endothelin-3 immunoreactivity was measured with a commercially available kit (Peninsula Laboratories). The sensitivity of this radioimmunoassay was 12.7 pg at 50% binding, and the intra-assay coefficient of variation was 5%. The antiserum used in this assay has only 2% cross-reactivity with endothelin-2 and 1% with endothelin.

Estimated changes in plasma volume were calculated according to the following formula: \(dV = \frac{100}{100 - H_f} \times \frac{100 \times (H_i - H_f)}{H_f}\), where \(dV\) is the percent change in plasma volume, and \(H_i\) and \(H_f\) are the initial and final hematocrit, respectively. Data are expressed as mean±SEM. Two-way analysis of variance and Student's \(t\) test were used to assess significance between and among groups. A value of \(p = 0.05\) was considered the minimum level of significance.

**Results**

**Effect of Endothelin on Arterial Pressure, Hematocrit, and Plasma Protein Concentration**

Infusion of endothelin induced a progressive increase in mean arterial pressure (MAP) by 1.7±1.3%, 6.8±1.5%, and 12.1±2.1% at 15, 30, and 45 minutes, respectively, from a basal value of 99±5 mm Hg (Figure 1). The increase in MAP had reversed by 30 minutes after discontinuation of the drug infusion. As also depicted in Figure 1, endothelin infusion induced a progressive time-dependent rise in hematocrit by 5.0±0.5%, 6.4±0.8%, and 8.0±0.6% at 15, 30, and 45 minutes, respectively, from a basal value of 43.4±0.8%. No clear return to preinfusion values was observed during the recovery period (+5.9±1.9% at the end of the recovery period). In the four rats with kidneys and spleens removed, endothelin increased MAP from 106±10 to 124±10 mm Hg (\(p<0.005\)) within 45 minutes. During the same time, hematocrit increased from 41.1±1.2% to 44±1.3% (+7.1±0.1%, \(p<0.005\)).

The decrease in plasma volume calculated from the change in hematocrit amounted to 13.1±0.9%, whereas it was only 2±1.3% for the vehicle. Such a decrease in plasma volume should have increased PPC by 16%, much greater than the observed increase of only 4.2%, suggesting that some loss of plasma protein may have occurred in response to endothelin infusion. To test this, we measured tissue accumulation of Evans blue dye in response to endothelin.

**Effect of Endothelin on Extravasation and Tissue Accumulation of Plasma Albumin**

The effects of vehicle or endothelin infusion on MAP and hematocrit observed in the animals injected with Evans blue were similar to those noted in

![Figure 1. Percent change in blood pressure (top panel) and hematocrit (bottom panel) during the experimental course in three groups of rats. O, vehicle (veh) infusion; ●, endothelin (ET) infusion; ▲, endothelin infusion in rats administered rabbit anti-rat ANP antiserum (ET + Ab); C, control value. *Values significantly greater than control; \(p<0.05\) by repeated measures analysis of variance.](http://hyper.ahajournals.org/)

![Figure 2. Percent change in hematocrit and plasma protein concentration after 45 minutes of vehicle (veh) (cross-hatched bars) or endothelin (ET) (solid bars) infusion. *Value after endothelin infusion significantly greater than vehicle, \(p<0.01\); + increase in hematocrit after endothelin significantly greater than increase in plasma protein concentration, \(p<0.05\).](http://hyper.ahajournals.org/)
corresponding groups depicted in Figure 1 (data not shown). As shown in Figure 3, the endothelin infusion resulted in significant accumulation of extravasated dye in cardiac and skeletal muscle and intestine, as compared with the vehicle group. In the liver (Figure 3), as well as in the other tissues studied (brain, lung, spleen, and testis; data not shown), no significant change in albumin extravasation was observed in response to endothelin infusion. These changes closely match those observed after infusion of ANP.13-14

**Effect of Endothelin Infusion on Plasma Concentrations of Endothelin and Atrial Natriuretic Peptide**

Basal values of MAP, hematocrit, and PPC, as well as the changes observed during endothelin infusion, were similar in this group of experiments to those noted in Figure 1 (Table 1). Infusion of endothelin (25 ng/kg/min for 45 minutes) resulted in a 3.3-fold increase in plasma concentration of endothelin (from a basal value of 86±11 pg/ml, p<0.01) and a parallel 3.7-fold rise in plasma concentration of ANP (from a basal value of 196±50 pg/ml, p<0.02). In addition, no change in right atrial pressure was observed during endothelin infusion. There was a significant correlation between the increase in endothelin immunoreactivity as a result of the infusion and the increase in plasma ANP concentration (Figure 4, r=0.84, p<0.01).

**Effect of Rabbit Anti-Rat Atrial Natriuretic Peptide Antiserum on the Response to Endothelin**

In six anesthetized rats, the mean diuretic, natriuretic, and kaliuretic response to a bolus injection of 1 μg rat ANP averaged 904±169%, 1,506±306%, and 231±97% of control, respectively (all p<0.05). Such responses were significantly reduced in the presence of rabbit anti-rat ANP antiserum (183±153%, 146±116%, and 38±26%, respectively; all p<0.01 when compared with the response observed in the absence of the antiserum), thus indicating the effectiveness of the antiserum.

Table 1. Effect of 45-Minute Infusion of Endothelin (25 ng/kg/min) on Hemodynamic Variables, Hematocrit, Plasma Protein Concentration, and Plasma Concentration of Endothelin and Atrial Natriuretic Peptide

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Endothelin infusion</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>103±5</td>
<td>119±8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>364±14</td>
<td>367±17</td>
<td>NS</td>
</tr>
<tr>
<td>Right atrial pressure (mm Hg)</td>
<td>1.7±0.3</td>
<td>1.4±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.0±0.7</td>
<td>45.3±1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma protein concentration (g/dl)</td>
<td>4.35±0.06</td>
<td>4.47±0.08</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma irET3 concentration (pg/ml)</td>
<td>86±11</td>
<td>285±43</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma irANP concentration (pg/ml)</td>
<td>196±50</td>
<td>722±203</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM of average values obtained in control and during endothelin infusion in 13 rats. The concentration of immunoreactive endothelin (irET3) in plasma was determined in nine rats. NS, not significant (p>0.05); irANP, immunoreactive atrial natriuretic peptide.
period. As depicted in Figure 1, when endothelin was infused into rats pretreated with the antiserum, MAP increased by 12.6±1.5%, 20.8±1.4%, and 25.3±2.9% at 15, 30, and 45 minutes, respectively, from a basal value of 95±4 mm Hg (p<0.002 versus endothelin infused in control rats). As shown in Figures 1 and 2, despite this larger rise in MAP, no increase in hematocrit and PPC occurred (+0.3±1.2% and +0.1±1.0% at 45 minutes, respectively; both not significant). In four rats pretreated with nonimmune rabbit serum, the effects of endothelin on MAP, hematocrit, and PPC were similar to those observed in the corresponding group depicted in Figures 1 and 2 (data not shown). Thus, pretreatment with anti-ANP antiserum entirely blocked the effect of infused endothelin to increase hematocrit and PPC but led to an exaggerated increase in MAP. Extravasation of albumin was not measured in this series of experiments.

Discussion

The results of our study confirm the effect of infused endothelin to increase hematocrit6,8,10 and provide clear evidence for the mechanism by which this action occurs. Because our experiments were carried out in animals that had undergone bilateral nephrectomy, negative fluid balance resulting from diuresis or natriuresis caused in some way by the endothelin infusion cannot account for our results. The spleen acts as a reservoir for red blood cells in some species, and splenic contraction, as induced by catecholamines, causes an increase in hematocrit that does not occur in splenectomized animals.19 We saw the same increase in hematocrit after endothelin infusion in four splenectomized rats, indicating that discharge of sequestered red blood cells from a contracted spleen could not be the basis for our results. The infusion of endothelin induced a modest increase in MAP (12%) as hematocrit rose. The peptide caused a much greater increase in pressure in rats administered antiserum against ANP; however, the antiserum eliminated the ANP-dependent increase in hematocrit, indicating that the increase in blood pressure, per se, could not be the basis for the observed hemoconcentration. Moreover, the increase in hematocrit also has been observed after the infusion of a nonpressor dose of endothelin,10 results we also have been able to confirm (unpublished observations from our laboratory).

On the other hand, our results do show that infused endothelin increases the vascular permeability to proteins and presumably to water. This initially was suggested by the observation that the increase in hematocrit, on a percentage basis, was much greater than the increase in PPC. The increase in hematocrit was caused by an estimated decrease in plasma volume of 13%. If this decrease in plasma volume was the result of loss of plasma water alone, then PPC should have increased by 16%. Instead, we observed an increase of only 4.2%, suggesting that proteins as well as water leaked out of the vascular tree in response to endothelin. This was confirmed in studies that measured the accumulation of Evans blue dye, bound to plasma albumin, in various organs after endothelin infusion. These studies indicated that endothelin promoted albumin extravasation in heart, skeletal muscle, intestine, and mesentery, but not in brain, liver, lung, or testis. Studies of endothelin administered to humans intravenously20 or intradermally21 have shown the development of a flare reaction, again suggesting an effect on vascular permeability.

This organ-specific pattern of albumin extravasation is identical to that observed as a result of ANP infusion13,14 and led us to question whether an endothelin-induced increase in plasma ANP concentration could account for our results. Endothelin infusion has been shown to increase plasma ANP concentration in in vivo studies4,5,8 and endothelin added to cardiac atrioocytes in vitro increases the release of ANP into the medium.6-8 In addition, specific binding of endothelin to cardiac tissue has been demonstrated.22 We found that a 45-minute endothelin infusion led to a nearly fourfold increase of immunoreactive ANP levels in plasma and that a correlation existed between the increase in plasma endothelin concentration observed as a result of the infusion and the increase in plasma ANP concentration (Figure 4). Although this increase in plasma ANP could have resulted from an endothelin-induced decrease in its metabolic clearance, given the aforementioned studies, it seems more likely to reflect an increase in ANP secretion. Our results therefore provide further evidence for a role of endothelin as a secretagogue for ANP in vivo and support the concept that the hemoconcentration and plasma protein extravasation seen after endothelin infusion could be mediated by the increase in plasma ANP concentration.

To test this directly, we carried out measurements of the vasopressor and hemoconcentrating effects of endothelin infusion in rats pretreated with rabbit antiserum raised against rat ANP. The antiserum was effective in vivo, because it markedly attenuated the diuretic, natriuretic, and kaliuretic actions of a large bolus of exogenously administered ANP. Infusion of endothelin in the animals given this antiserum had two striking effects. First, no change in hematocrit or PPC occurred over the time course of these experiments; indeed, the effects on hematocrit were nearly superimposable on the trivial changes seen in rats receiving vehicle alone (Figure 1). This observation provides telling evidence that the effect of endothelin to increase vascular permeability and cause hemoconcentration is not a direct result of the peptide itself but rather an indirect one due to its effect to stimulate the secretion of ANP and thereby increase its plasma concentration. Second, the same rate of endothelin infusion caused a much more pronounced increase in MAP (Figure 1) in the presence than in the absence of the antiserum. The most straightforward interpretation of this finding is that the potent vasoconstrictor action of endothelin is buffered in vivo by the endothelin-dependent release of the
blood-pressure-lowering peptide ANP. As such, it suggests an antagonistic relation between the two peptides that could represent a potentially important aspect of neurohumoral integration with respect to circulatory regulation and the control of body fluid homeostasis. Additional evidence from other laboratories supports such a relation. Infusion of ANP into rats blocks the pressor response to endothelin and minimizes the vasoconstrictor actions of endothelin on renal hemodynamics and on urine flow. In addition, subpressor endothelin infusions may produce a natriuresis that is prevented by the prior administration of specific anti-ANP antibodies, suggesting that, as in our studies, some of these observed effects of endothelin may reflect secondary stimulation of ANP release. Taken together, these data suggest that endothelin does mediate ANP secretion and that this secreted ANP may represent a counter-regulatory mechanism that is activated to buffer the vasoconstrictor activity of the pressor peptide.

In summary, infusion of a mildly pressor dose of endothelin elicits an increase in the concentration of ANP in plasma and an ANP-mediated increase in vascular permeability and decrease in plasma volume. Further study of the interaction of these two vasoactive peptides should clarify understanding of their roles in the control of the circulation in vivo.

Acknowledgments

We thank Sue Montgomery, RN, who provided technical assistance, and Annalissa Spiers for administrative assistance.

References


Key Words: vasodilation • plasma volume • capillary permeability • extravasation
Modulation of endothelin effects on blood pressure and hematocrit by atrial natriuretic peptide.
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Hypertension. 1991;17:864-869
doi: 10.1161/01.HYP.17.6.864

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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