Platelet Binding Sites for Atrial Natriuretic Factor in Humans
Characterization and Effects of Sodium Intake

ERNESTO L. SCHIFFRIN, MARIO DESLONGCHAMPS, AND GAÉTAN THIBAULT

SUMMARY Platelets bear receptors for vasoactive peptides such as angiotensin II and vasopressin. The presence of binding sites for another vasoactive peptide, atrial natriuretic factor, was therefore investigated in human platelets. Iodine-125-labeled synthetic atrial natriuretic factor bound specifically to human platelets. Steady-state and kinetic experiments demonstrated the existence of one class of high-affinity low-capacity binding sites for atrial natriuretic factor in platelets with a dissociation constant of 30 pM. The order of potency of atrial natriuretic factor fragments showed that the structural requirements of the high-affinity binding site detected were similar to those of receptors for atrial natriuretic factor in rat blood vessels and adrenal zona glomerulosa. To study the regulation of these binding sites by sodium, normal young men were subjected successively in random order to a low-sodium (40 mmol per day) and high-sodium (300 mmol per day) diet for 4 days. Binding of atrial natriuretic factor to platelets was higher with the low-sodium diet (10.3 ± 1.0 sites per cell) than with the high-sodium diet (7.1 ± 0.7 sites per cell). In conclusion, human platelets bear binding sites for atrial natriuretic factor, the density of which may be modulated by sodium intake. The platelet is a useful model for investigating atrial natriuretic factor receptors in different physiopathological conditions in humans. (Hypertension 8 [Suppl II]: II-6-II-10, 1986)

KEY WORDS • atrial natriuretic factor receptor regulation • platelets • sodium balance

THE atria of different species have been shown to possess a potent diuretic and natriuretic activity. The family of peptides isolated from atria with this property has been collectively termed atrial natriuretic factor (ANF), although terms such as atriopeptin, auriculin, cardionatrin, and natriodilatin are also used to designate some of them. These peptides have been purified and their sequence is now well known. ANF has been shown to possess powerful vasorelaxant activity and to be a potent inhibitor of aldosterone secretion.

Receptors for ANF have recently been characterized in rabbit aorta and renal tubular cells and in rat adrenal glomerulosa and vasculature. Since platelets bear receptors for vasoactive peptides, such as angiotensin II and vasopressin, and α2-adrenergic receptors, all of which are found in the extrasynaptic smooth muscle cell membrane, we decided to search for ANF binding sites in human platelets. These might become a useful tool for examining the regulation of ANF receptors in humans. This report documents the presence of ANF binding sites in human platelets, characterizes the binding, and describes the changes occurring after sodium loading in normal human volunteers.

Materials and Methods

Materials

Synthetic ANF (Arg 101–Tyr 126) was generously provided by Dr. R.F. Nutt of Merck Sharp and Dohme Research Laboratories (West Point, PA, USA). Human ANF (99–126), ANF (103–123) or atriopeptin I, ANF (103–125) or atriopeptin II, ANF (103–126) or atriopeptin III, human angiotensin II, and arginine-vasopressin were obtained from Peninsula (San Carlos, CA, USA).

Iodination of Atrial Natriuretic Factor

Synthetic rat ANF (101–126) or human ANF (99–126) was iodinated with iodine-125 by the lactoperoxidase method. In brief, 5 μg of ANF was exposed to 1
mCi of 125I-labeled sodium (New England Nuclear, Boston, MA, USA) in the presence of 5 μg of lactoperoxidase (Sigma Chemical, St. Louis, MO, USA) in a total volume of 80 μl of 0.05 M sodium phosphate buffer, pH 7.4. The reaction proceeded after the addition of 10 μl of 0.6% hydrogen peroxide (vol/vol) at 5-minute intervals three times. Radiolabeled ANF was separated by high-pressure liquid chromatography on a C18 /iBondapak column eluted with a linear gradient of 20 to 45% acetonitrile and 0.1% trifluoroacetic acid, with a slope of 0.5%/min and a flow rate of 1 ml/min. Specific activity, measured by radioreceptor assay and self-displacement, was approximately 1000 Ci/mmol.

Preparation of Platelets

The method used was essentially the same as that described by Ding et al.13 Peripheral venous blood (45 ml) was collected into plastic tubes containing 13 mM sodium citrate, 2.2 mM citric acid, 2.2 mM sodium biphosphate, 0.3 mM adenine, and 5 mM dextrose (final concentration in the blood). Blood was carefully layered on an isosmotic solution of Percoll (specific gravity, 1.06) from Pharmacia Fine Chemicals (Uppsala, Sweden) and centrifuged at 400 g for 5 minutes at 4°C. The upper platelet-rich layer was aspirated; washed twice with an equal volume of 135 mM sodium chloride, 13 mM sodium citrate, 1 mM ethylenediaminetetraacetic acid, and 5 mM glucose, pH 6.5; and centrifuged at 1000 g for 10 minutes at 4°C. Finally, the platelets were resuspended in medium 199 in 1 ml containing 0.5 mM phenylmethylsulfonyl fluoride, 0.1% bacitracin, 1 μM aprotinin, and 0.4% bovine serum albumin were added.

Binding Assay

Aliquots of platelet preparation (200 μl) containing 5 × 10^4 platelets per microliter were incubated in plastic tubes with fixed concentrations of labeled ANF (approximately 20 pM) and increasing concentrations of unlabeled peptides in competition experiments. In saturation experiments, platelets were incubated with increasing concentrations of labeled ANF (0.6-100 pM), and nonspecific binding was measured in the presence of 10^-10 M ANF. The final volume was 250 μl. Platelets were incubated at 22°C for 60 minutes under constant agitation. Separation of bound and free radioactivity was performed by rapid filtration through Whatman GF/C filters soaked previously with 0.5 ml of assay buffer. Filters were washed twice with 3 ml of assay buffer, then allowed to dry and counted in a Rackgamma LKB counter (Turku, Finland) with 65% efficiency.

Separation of Red and White Cells

Binding of ANF to red and white blood cells was studied, since 0.5% contamination with these cells was present in the platelet preparation. Red blood cells were obtained during centrifugation of blood for platelet isolation. Cells were washed with 0.9% sodium chloride before resuspension in assay medium. White blood cells were separated on a preformed Percoll gra-

Subjects and Protocol for Study of Sodium Intake

Five normal male volunteers (physicians and laboratory technicians) aged 24 to 38 years gave written informed consent to participate in the study, which had previously been approved by the Ethics Committee of the Clinical Research Institute of Montreal. On an ambulatory basis, they were put on a 4-day low- or high-sodium diet in random order. During the low-sodium period, their sodium intake was 40 mmol per day, in a diet also providing 90 mmol of potassium per day. During the high-sodium period, approximately 260 mmol of sodium chloride was added each day. A period of 3 days with a sodium intake of 135 mmol per day separated the low- and high-sodium diets. On the morning of the 5th day of the diet, the subjects arrived at the laboratory at 0800, and blood sampling for platelets, plasma renin activity, aldosterone, and electrolyte concentrations was performed after the subjects had been in the recumbent position for 15 minutes. Twenty-four-hour urine samples were collected on the last 2 days of each diet for measurement of sodium, potassium, and creatinine.

Biochemical Methods

Plasma renin activity and aldosterone concentrations were measured by radioimmunoassay.17 18 Electrolytes and creatinine were measured by standard methods.

Analysis of Data

Results are expressed as means ± SEM. Statistical comparisons were performed with Student's t test. Binding data were analyzed by computer-assisted nonlinear regression analysis with the LIGAND program19 to determine the density and affinity of binding sites. The ALLFIT program,20 based on the four-parameter logistic equation, was used to calculate the concentration of unlabeled ANF and related peptides inhibiting binding by 50% in competition experiments.

Results

The specific binding of ANF to the human platelet preparation is depicted in Figure 1. High-affinity binding sites with a density of 9.8 ± 1.4 sites per platelet (n = 4) and a dissociation constant of 25 ± 8 pM were demonstrated in both competition experiments (see Figure 1) and saturation experiments (Figure 2). Binding was linear between platelet concentrations of 0.2 and 1.5 × 10^6 platelets per tube, and in all experiments reported 1 × 10^6 platelets per tube were employed (Figure 3). No binding of ANF could be detected on red or white blood cells at a concentration of 2 × 10^6 cells per tube, equivalent to a 2% contamination of the platelet preparation.

Degradation of ANF in the incubation medium was
Figure 1. Representative competition curve of the binding of $^{125}$I-labeled atrial natriuretic factor (ANF) to human platelets. $^{125}$I-ANF (20 pM) was incubated with increasing concentrations of unlabeled ANF ($10^{-12}$-$10^{-6}$ M) and $1 \times 10^8$ platelets per tube in a final volume of 0.25 ml. Results were fitted with the computer-supported LIGAND program and represented as bound/total (B/T) versus total ligand (labeled + unlabeled). The maximum number of binding sites ($B_{\text{max}}$) was 7.2 per cell. $K_d$ = dissociation constant.

Figure 2. Representative saturation curve of the binding of $^{125}$I-labeled atrial natriuretic factor (ANF) to human platelets. The Scatchard analysis is depicted in the right panel. Increasing concentrations of $^{125}$I-ANF (0.6-100 pM) were incubated with $1 \times 10^8$ platelets per tube in 0.25 ml, with or without $10^{-8}$ M ANF, to determine total binding (○), nonspecific binding (●), and specific binding (▲). Specific binding in the Scatchard plot was determined with the LIGAND program after fitting the binding data, including displaceable nonspecific binding, as data-determined unknown parameters. $B_{\text{max}}$ = maximum number of binding sites; $K_d$ = dissociation constant.

examined by reverse-phase high-pressure liquid chromatography. In the presence of bacitracin, aprotinin, and phenylmethylsulfonyl fluoride, more than 90% of the labeled peptide was intact after a 60-minute incubation with platelets.

Using N-terminal and C-terminal truncated ANF fragments, we characterized the platelet receptor for ANF and were able to compare the relative potency of these peptides with that observed in the rat vasculature. Figure 4 shows the competition of these different peptides with labeled ANF for binding to human platelets. The splicing at the N-terminal or C-terminal produced a marked drop in potency. These results are in agreement with the results of a rat vascular ANF binding assay (Table 1). Human ANF was as potent as rat ANF in this assay. When the labeled ligand was human ANF, results were similar to those obtained with rat ANF (see Table 1).

There was no displacement of $^{125}$I-labeled ANF from the platelet preparation by unrelated peptides known to bind to platelets such as angiotensin II or vasopressin up to a concentration of 1 μM.

To determine whether ANF binding sites could be regulated by changes in dietary sodium intake, 5 normal male human volunteers were put on a diet containing either 40 or 300 to 350 mmol of sodium and 90 mmol of potassium per day for 4 days in random order, with an intermediate period of 3 days on a diet of 135 mmol of sodium and 90 mmol of potassium per day.

The changes in blood pressure, weight, plasma electrolytes, renin activity, and aldosterone are shown in Table 2. The expected suppression of plasma renin activity and reduction of plasma aldosterone concen-
Discussion

This report demonstrates the presence of high-affinity binding sites for \( ^{125}\text{I}-\text{ANF} \) in human platelets and their regulation by sodium intake. These binding sites have structural requirements similar to those of vascular receptors in the rat.\(^{11}\) We have previously demonstrated that ANF vascular receptors in rats are down-regulated by sodium loading.\(^{22}\) Since these human platelet binding sites for ANF resemble rat vascular ANF receptors in their structural requirements and regulation, they may serve as a model of the changes occurring in human vascular ANF receptors.

Plasma levels of ANF rise when human subjects increase their intake of sodium chloride.\(^{21}\) Indeed, as part of our study of sodium intake, plasma levels of ANF were measured in four of the five volunteers on both diets and are reported elsewhere.\(^{21}\) Plasma ANF was 40\% higher on the high-sodium diet (a mean of 24 fmol/ml vs 17 fmol/ml on the low-sodium diet). This raises the possibility that prior occupancy of receptors gives the appearance of decreased receptor density or that actual down-regulation of ANF binding sites occurs after sodium loading. Our results with cold or labeled ANF added before purification of platelets suggest that prior occupancy does not account for decreased binding after sodium loading. We have observed down-regulation of ANF receptors by ANF in cultured smooth muscle cells.\(^{23}\) As with platelets, when these cultured cells were exposed to labeled ANF, after the same washing procedure used for the binding assay, no radioactivity could be recovered, thus effectively ruling out prior receptor occupancy.

If down-regulation does occur as a response to increased ambient ANF after sodium loading, it occurs fairly rapidly, within 4 days of increased sodium intake. Decreased synthesis, increased degradation, or the inaccessibility of receptors to the ligand could account for decreased binding. Changes in synthesis are unlikely, since platelets have little protein synthetic capacity. Newly released platelets are more active than older ones, however, and considering the short life span of platelets, this might account in part for the

### Table 1. Potency of Atrial Natriuretic Factor Peptides Competing with Labeled Human or Rat Atrial Natriuretic Factor for Binding to Human Platelets or to Rat Mesenteric Artery

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Human platelets</th>
<th>Rat mesenteric artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ^{125}\text{I}-\text{hANF} )</td>
<td>36</td>
<td>137</td>
</tr>
<tr>
<td>( ^{125}\text{I}-\text{rANF} (99-126) )</td>
<td>58</td>
<td>170</td>
</tr>
<tr>
<td>( ^{125}\text{I}-\text{rANF} (101-126) )</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>( ^{125}\text{I}-\text{rANF} (103-126) )</td>
<td>350</td>
<td>2900</td>
</tr>
</tbody>
</table>

Results are means of two to four experiments.

Rat mesenteric artery was prepared by a modification of methods described elsewhere.\(^{11}\) \( K_i \) = inhibition constant; ANF = atrial natriuretic factor; h = human; r = rat.

### Table 2. Effect of Low- and High-Sodium Diets on Clinical and Humoral Parameters and Binding of Atrial Natriuretic Factor to Platelets in Five Normal Human Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low sodium</th>
<th>High sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>61.1 ± 3.5</td>
<td>62.8 ± 3.5*</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>114.5 ± 2.67/15.4 ± 4.2</td>
<td>115.0 ± 2.9/68.5 ± 4.3</td>
</tr>
<tr>
<td>Urinary sodium (mmol/day)</td>
<td>61.6 ± 6.8</td>
<td>353.6 ± 19.1*</td>
</tr>
<tr>
<td>Urinary potassium (mmol/day)</td>
<td>66.5 ± 4.2</td>
<td>83.0 ± 7.8</td>
</tr>
<tr>
<td>Plasma renin activity (ng·ml(^{-1}·)hr(^{-1}))</td>
<td>1.17 ± 0.32</td>
<td>0.25 ± 0.08*</td>
</tr>
<tr>
<td>Plasma aldosterone (ng·dl(^{-1}))</td>
<td>22.2 ± 4.2</td>
<td>12.5 ± 2.7*</td>
</tr>
<tr>
<td>Serum sodium (mmol·L(^{-1}))</td>
<td>141.0 ± 0.7</td>
<td>141.5 ± 0.5</td>
</tr>
<tr>
<td>Serum potassium (mmol·L(^{-1}))</td>
<td>3.98 ± 0.13</td>
<td>3.90 ± 0.08</td>
</tr>
<tr>
<td>ANF binding capacity (sites/platelet)</td>
<td>10.3 ± 1.0</td>
<td>7.1 ± 0.7*</td>
</tr>
<tr>
<td>( K_i ) (pM)</td>
<td>34 ± 6</td>
<td>34 ± 9</td>
</tr>
</tbody>
</table>

Results are means ± SEM.

ANF = atrial natriuretic factor; \( K_i \) = dissociation constant.

\(*p < 0.01\) (paired t test).
change observed, as suggested for angiotensin II receptors on human platelets. The mechanism responsible for these changes remains to be elucidated.

The importance of ANF binding sites in platelets is not clear. ANF does not appear to modulate platelet function. In a recent study ANF had no effect on ADP- or thrombin-induced aggregation or on platelet function. In another study ANF had no effect on platelet aggregation induced by adenosine 5'-diphosphate (ADP) at ANF concentrations of 1 μg/ml has recently been reported, but this is unlikely to be related to a physiologically marked effect on platelet function. In another study ANF had no effect on platelet function, although platelet an

dependent monophosphate, a putative mediator of ANF action, weak effect on platelet aggregation induced by adenosine 5'-diphosphate (ADP) at ANF concentrations of 1 μg/ml has recently been reported, but this is unlikely to be related to a physiologically marked effect on platelet function. In another study ANF had no effect on platelet function, although platelet an

The density of binding sites for ANF on platelets is small (on the order of 10 sites per platelet) or 10^7 per 15 fmol per 10^6 platelets). The number of ANF sites on platelets is thus less than 5% of the density detected on smooth muscle cells, per milligram of protein. The density is similar, however, to that of angiotensin II binding sites on platelets. Whether the low density of ANF binding sites relates to the lack of biological response to ANF found in platelets remains to be determined.

In summary, we have documented and characterized ANF binding sites in human platelets and their regulation by sodium intake. The availability of platelets makes this system suitable for studying ANF receptors in humans.

Acknowledgments

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