Rat Platelets Activate High Molecular Weight Atrial Natriuretic Peptides in Vitro
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SUMMARY Evidence suggests that the more biologically active low molecular weight forms (<10,000) of rat atrial natriuretic peptides are proteolytically derived from a less active precursor of higher molecular weight. Conversion and activation could occur within the myocyte as well as during circulation. The present study found that in vitro rat blood and platelets were capable of converting the high molecular weight atrial natriuretic peptides (>10,000) to low molecular weight atrial natriuretic peptides within minutes and that enhanced biological activity attended the conversion. Rat high molecular weight peptides were partially purified by gel filtration, lyophilized, and reconstituted in Krebs-Ringer bicarbonate buffer. One milliliter of fresh rat blood was incubated with the high molecular weight peptides at 37 °C for 2 minutes. After centrifugation, the supernatant was fractionated on Sephadex G-75. Natriuretic activity was determined by bioassay in anesthetized rats. In contrast to the results following incubation of high molecular weight peptides in Krebs-Ringer bicarbonate buffer alone, which showed that 95% of the natriuretic activity remained in the high molecular weight peptide region, the natriuretic activity of the blood-treated high molecular weight peptides eluted almost exclusively in the low molecular weight peptide region, which indicates conversion. Blood was separated into plasma, erythrocytes, lymphocytes, and platelets. Conversion of high to low molecular weight peptides occurred only after incubation with platelets. Compared with control high molecular weight peptides, rat platelet-treated high molecular weight peptides had significantly greater activity in relaxing histamine-contracted rabbit aortic smooth muscle (p < 0.05). Heparin inhibited rat blood-associated and platelet-associated conversion of high molecular weight peptides and platelet-induced activation of aortic relaxant activity of high molecular weight peptides. These results suggest that if high molecular weight atrial natriuretic peptides are released or injected into the circulation, rat platelets might play a role in their activation by converting them to low molecular weight atrial natriuretic peptides. (Hypertension 7: 905-912, 1985)

KEY WORDS • atrial natriuretic factor • conversion • activation • vasorelaxation • natriuresis

The rat atrial natriuretic peptides (rANP) are composed of multiple forms that have amino acid sequences differing in length. The number of residues that have been determined or deduced in each peptide so far vary from 21 to 126, which confirms earlier gel filtration results showing that natriuretic activity eluted in high and low molecular weight regions. To explain the existence of both high and low molecular weight forms of rANP, a precursor-product relationship was suggested. The precursor concept was supported by the structural similarities of the rANP (i.e., various truncated versions) and by the observation that low molecular weight atrial natriuretic peptides (LMWP) could be derived from high molecular weight rANP (HMWP) by in vitro proteolysis. Moreover, rat atrial extracts were found to contain a heat-labile factor that was capable of converting HMWP to LMWP. Recently, the structure of rANP precursor was determined by cloning its complementary DNA and found to contain the amino acid sequences of all rANP reported to date. These findings leave little doubt that rANP of different lengths are derived from the precursor by variable proteolytic cleavages either in vivo as natural processing or in vitro as artifacts resulting from the extraction and purification procedures, or both.

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The purified HMWP have less natriuretic and smooth muscle relaxant activities than LMWP, and conversion of partially purified HMWP to LMWP by in vitro proteolysis results in increased biological activity. These findings suggest that the precursor must be converted to LMWP to be fully biologically active. In vivo conversion and activation of rANP may occur during processing and storage within the atrial myocyte, during release, or at an extracellular site (or sites). The presence of a heat-labile converting factor in rat atrial extracts supports the concept that conversion and activation occur within the myocyte or during release. However, there is also evidence suggesting extracellular processing of rANP. Injection of HMWP into the renal artery of anesthetized rats caused little renal vascular action; however, when HMWP were administered intravenously renal vascular resistance decreased, which suggests that activation had occurred during circulation.

It is possible that proteases capable of converting HMWP to the more biologically active LMWP are present within the lining of blood vessels in certain organs or, perhaps, are present in the blood. The purposes of this study were to determine whether conversion of rat HMWP to LMWP could occur in rat blood and, if so, whether activation or enhanced biological activity attended the conversion.

Materials and Methods

Solutions

Krebs-Ringer bicarbonate buffer (KRBB), pH 7.6, contained (in g/L) NaCl, 6.54; NaHCO₃, 2.14; KCl, 0.35; CaCl₂, 0.28; MgCl₂, 6H₂O, 0.24; KH₂PO₄, 0.16; glucose, 2.07; and ethylenediaminetetraacetic acid (EDTA), 0.01. Phosphate-buffered saline, pH 7.2, contained (in g/L) NaCl, 8.0; Na₂HPO₄, 1.15; KCl, 0.2; KH₂PO₄, 0.2; MgCl₂, 0.04.

Preparation of Rat Atrial Natriuretic Peptides

Partially purified rANP were prepared as described. Briefly, atria from Sprague-Dawley rats were homogenized in 1.0 M acetic acid, centrifuged, and lyophilized. The extract was homogenized in 1.0 M acetic acid and centrifuged; the supernatant was fractionated on Sephadex G-75 in 1.0 M acetic acid. The HMWP eluted in fractions representing 10,000 to 25,000, and the LMWP at approximately 2,000 to 10,000 daltons. The fractions containing HMWP and LMWP were combined separately and lyophilized in aliquots, which were stored at −70 °C until used. Protein was determined by the method of Lowry et al., and natriuretic activity was measured according to the bioassay described in the next section. Specific activities of the HMWP and LMWP were 75 and 100 natriuretic units/mg protein, respectively.

Natriuretic Bioassay

Male Sprague-Dawley rats (150–200 g) were anesthetized with an intraperitoneal injection of 100 mg/kg of thiobutabarbital (Inactin; BYK Gulden Konstanz, West Germany). Catheters were inserted into the trachea, femoral vein, and urinary bladder as described. After a 30-minute stabilization period, urine was collected during a 30-minute control period. The test substance was dissolved in phosphate-buffered saline and injected intravenously at 4 ml/kg. Urine was collected during a 10-minute test period. Twenty minutes after the test period, a second 30-minute control period was started and the rats were injected with a second test substance. Previous studies have indicated that the first injection does not alter the rats’ responsiveness to the second injection. Urine volume was determined by weight, and sodium concentration by flame photometry. As reported, units of natriuretic activity were determined from the empirical log dose-response line: log natriuretic unit = (Ru − 20)/510, where Ru is the change in urinary sodium excretion (response in microequivalents per kilogram of body weight per 10 min) during the 10 minutes following injection, 20 is the response to one defined unit, and 510 is the slope. Samples eliciting responses below the minimal detectable response of 15 μEq/kg/10 min were considered inactive. Samples eliciting responses in the maximum range (≥400 μEq/kg/10 min) were diluted for further analysis. Whether a response fell within the minimum or maximum range was estimated on a daily basis from the urine volume; however, the final determination could only be made after the sodium concentration was measured, usually several days after each sample was assayed. For this reason, the number of assays used to obtain the average natriuretic units in each sample varied from two to five. All final values were corrected for the actual volume injected and for any dilutions that were made.

Blood and Blood Components

Fresh blood containing heparin (50 U/ml), EDTA (1.5 mg/ml), or no anticoagulants was obtained from anesthetized rats by percutaneous cardiac puncture. Washed erythrocytes were obtained from blood containing EDTA. To obtain platelets, 4 ml of blood that contained 6 mg of EDTA was centrifuged at 140 g for 20 minutes at room temperature. Platelet-rich plasma, obtained from the supernatant, was centrifuged at 850 g for 20 minutes at room temperature. The platelet pellet recovered from 0.2 ml of platelet-rich plasma was used for each experiment for incubation with rANP. Rat lymphocytes were obtained from 10 ml of blood-containing EDTA using Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ, USA). In preliminary experiments, a few drops of the preparations containing lymphocytes or platelets were smeared on glass slides, stained with Wright’s stain, and viewed with a microscope to ensure that each technique yielded preparations with a high proportion of the desired cell type.

Incubation

The rANP were incubated with blood or blood components and fractionated to determine if conversion had occurred or was tested for biological activity to determine if activation had occurred. Quantities for each experiment were as follows: 1 ml of blood, 1 ml...
of plasma, 0.2 ml of washed erythrocytes, lymphocytes harvested from 10 ml of blood, and platelets harvested from 0.2 ml of platelet-rich plasma. For each fractionation experiment 200 natriuretic units, unless indicated otherwise, of rat HMWP was dissolved in 2.5 ml of KRBB and warmed to 37 °C. The HMWP-KRBB mixture was added to the blood or blood component and mixed by inversion. Platelets were suspended by gently aspirating the pellet in a plastic pipette several times. The mixture was incubated for 2 minutes at 37 °C. During incubation the mixture was periodically mixed by hand to maintain suspension of particles. After the mixture had been centrifuged at room temperature for 5 minutes, the volume of supernatant was brought to 4 ml with 0.1 M acetic acid; this mixture was immediately placed on a Sephadex G-75 column equilibrated in 0.1 M acetic acid. The fractions, eluted in 0.1 M acetic acid, were lyophilized and dissolved in 2 ml of phosphate-buffered saline for bioassay of natriuretic activity. In one experiment 200 natriuretic units of HMWP was incubated in 3.5 ml of KRBB only (2.5 ml of HMWP-KRBB mixture plus 1 ml of KRBB instead of blood) and then fractionated as described.

For each experiment for the testing of biological activity on rabbit aortic rings, 17 natriuretic units of HMWP or LMWP was dissolved in 1.7 ml of KRBB. The rANP-KRBB mixture was divided into two 0.7-ml portions and warmed to 37 °C. One portion was added to the platelet pellet recovered from 0.2 ml of platelet-rich plasma. The platelets were suspended in the rANP-KRBB mixture by gently aspirating the pellet in a plastic pipette several times. The platelet-rANP-KRBB suspension was incubated at 37 °C for 2 minutes and was mixed periodically by hand. At the end of the incubation period, the mixtures were immediately centrifuged at 40,000 g at 4 °C for 10 minutes and the supernatants were kept in ice. Vasorelaxant activity was determined in the supernatants on the same day using contracted rabbit aortic rings.

Rabbit Aortic Rings

Domestic rabbits were stunned by a blow to the head and killed by exsanguination. Thoracic aortic rings (4 mm long) were placed in an organ bath containing 30 ml of KRBB, bubbled with 95% O₂, 5% CO₂, and maintained at 37 °C. A resting force of 1.5 g was applied, and the rings were allowed to equilibrate for 90 minutes. Force was measured and recorded with Grass transducers and recorder (Quincy, MA, USA). The rings were contracted to 90% maximum for 15 minutes with 3 μM histamine. The bath was rinsed three times with KRBB, and after 30 minutes, the rings were contracted a second time. At 15 minutes, the test substance was added and the rings were allowed to respond for 20 minutes. Relaxation responses were taken as the difference between the force (g) at the plateau of contraction and the least force measured during the 20-minute response period. Each ring was used for only one dose of test substance, unless indicated otherwise.

Statistical Analysis

Analysis of variance and, when appropriate, the Newman-Keuls test were used for statistical analysis. A p value of 0.05 or less was considered significant. Means ± se are given.

Results

When the HMWP were incubated in KRBB alone and then fractionated in 0.1 M acetic acid, natriuretic activity remained in the 10,000- to 25,000-dalton range (Figure 1A). This response indicated that the processes of dissolving the HMWP in KRBB, incubating them briefly at 37 °C, and refractionating them in 0.1 M acetic acid had no effect on their molecular weights. This control experiment was not repeated, since this mild treatment was not expected to cause any molecular weight changes in the HMWP. Indeed, in the instances in which the blood components had no effects on molecular weight, such as with erythrocytes, lymphocytes (Figure 2B), and boiled platelets (Figure 2C), the peak natriuretic activity eluted at a volume very similar to that in the control experiment (Figure 1A). However, when the HMWP were incubated with rat blood and then fractionated, natriuretic activity was found almost exclusively in the less than 10,000-dalton range, which indicates conversion to LMWP. The results of three such experiments, two with blood containing no anticoagulants and one with blood containing EDTA, are shown in Figure 1B. A fourth experiment with whole blood was conducted by incubating 100 natriuretic units with blood containing no anticoagulants. The HMWP (10,000–25,000) and the LMWP (~2,000–10,000) fractions were combined separately. After bioassay, we found only 8 natriuretic units remaining in the HMWP fraction and 40 natriuretic units in the LMWP fraction. These results indicate that the blood contained a factor that was capable of converting HMWP to LMWP.

When the HMWP were incubated with blood containing heparin there was only a slight shift of natriuretic activity toward the lower molecular weight range. Two such experiments are shown in Figure 1C. Since very little natriuretic activity appeared in the less than 10,000-dalton range, these results suggest that heparin inhibited the blood conversion reaction. There was an approximate 50% reduction in recovery of total natriuretic activity after incubating HMWP with blood containing heparin (Figure 1C) as compared with incubation in KRBB alone (Figure 1A).

The results of two experiments with plasma, one with heparinized plasma and the other with an EDTA-plasma mixture, are shown in Figure 2A. There was a slight shift in natriuretic activity toward the lower molecular weight range when the HMWP were incubated in rat plasma; however, the major amount of natriuretic activity eluted at less than 10,000 daltons (<100 ml). Thus, although some proteolysis may have occurred in the plasma, the definite conversion to the less than 10,000-dalton range, as observed with rat whole blood (Figure 1B), was not evident.

The HMWP were incubated with washed erythro-
Figure 1. Analysis of rat high molecular weight atrial peptides (HMWP) by Sephadex G-75 fractionation in 0.1 M acetic acid. Before fractionation, HMWP (200 natriuretic units) were dissolved in Krebs-Ringer bicarbonate buffer (KRBB) and incubated for 2 minutes at 37 °C with the following constituents: (A) KRBB alone; (B) 1 ml of rat blood containing no anticoagulants (circles; 2 experiments with blood from 2 different rats) or 1 ml of rat blood containing ethylenediaminetetraacetic acid (squares); (C) 1 ml of rat blood containing heparin (2 experiments with blood from 2 different rats). Natriuretic units were determined from the change in urinary sodium excretion in anesthetized assay rats using the following empirical log dose-response relationship: log unit = (Ru - 20)/510, where Ru is the response (change in urinary sodium excretion; microequivalents per kilogram body weight per 10 min) during the 10 minutes following injection of the unknown sample, 20 is the response to 1 defined natriuretic unit, and 510 is the slope of the log dose-response line. The peak elution volumes (and molecular weights) of aldolase (158,000), ovalbumin (43,000), chymotrypsinogen A (25,000), ribonuclease A (13,700), and 22Na were 48 ml, 66 ml, 77 ml, 91 ml, and 147 ml, respectively.

cytes in one experiment and with lymphocytes in another experiment. As shown in Figure 2B, little, if any, conversion of HMWP to LMWP resulted from incubation with these formed elements. These results suggest that the large shift in the molecular weight of HMWP caused by incubation in whole blood, as demonstrated by the results in Figure 1B, probably resulted from some component of blood other than the erythrocytes and lymphocytes.
HMWP for incubation with preheated platelets, we combined the HMWP and LMWP fractions separately after gel filtration. We found 27 natriuretic units in the HMWP fraction and only 7 natriuretic units in the LMWP fraction. These results suggest that the platelet-associated converting factor for HMWP was destroyed by heat. There were variable recoveries of total natriuretic activities in these experiments (Figure 2). Since the presence of heparin in whole blood inhibited the converting activity, one experiment was conducted to determine if heparin inhibited the converting activity associated with platelets. Rat platelets obtained from 0.2 ml of platelet-rich plasma were incubated with 100 natriuretic units of HMWP in 2.5 ml of KRBB containing 50 units of heparin per milliliter. After incubating for 2 minutes at 37 °C, the mixture was centrifuged and the supernatant was fractionated as already described. The HMWP and LMWP fractions were combined separately, lyophilized, and tested for natriuretic activity. We found 22 natriuretic units in the HMWP fraction and only 5 natriuretic units in the LMWP fraction, which indicated that heparin inhibited the platelet converting activity.

To determine if the platelet-induced conversion of HMWP to LMWP was associated with enhanced biological activity, we measured the vasorelaxant activity of HMWP that had been incubated in KRBB alone or in KRBB plus rat platelets. The platelet-HMWP incubation was replicated in five separate experiments, and the concentration-response relationship was determined in a cumulative fashion. Control experiments were conducted by incubating the platelets in KRBB alone. A control experiment (HMWP plus KRBB alone) was performed along with each platelet-HMWP incubation. After incubation, the mixtures were centrifuged and the supernatants were tested for vasorelaxant activity at three dosage levels by adding them to 30-ml baths containing rabbit aortic rings contracted with 3 μM histamine. The dosage levels were 5, 10, and 20 μl, representing 0.625, 1.25, and 2.5 μg of preincubation HMWP protein, respectively. Each aortic ring was used for only one dose and was discarded. In two of the five experiments, both the control and the platelet-incubated HMWP were added to two sets of aortic rings, so that the number of observations shown in Figure 3 is seven for each point. The relaxation caused by the platelet-incubated HMWP was significantly greater than that caused by the HMWP-incubated HMWP at all three doses (Figure 3). These results indicate that the biological activity of the HMWP was enhanced by platelet incubation.

To determine if platelet incubation would enhance the biological activity of LMWP, we conducted similar experiments with the less than 10,000-dalton fraction of partially purified atrial extract. The same protocol used for HMWP experiments was used for the LMWP experiments. Five replicate experiments (incubations of both control and platelet-LMWP) were performed. In two of the experiments, vasorelaxant activity was tested on two sets of aortic rings, so that the number of observations shown in Figure 4 is seven for each point. The relaxation caused by the platelet-incubated LMWP was not significantly different from that caused by the KRBB-incubated LMWP (Figure 4). These results suggest that the biological activity of LMWP was not altered by platelet incubation.

To determine if heparin inhibited the platelet-induced enhancement of vasorelaxant activity of HMWP, rat platelets obtained from 0.2 ml of platelet-rich plasma were incubated with 7 natriuretic units of HMWP in 0.7 ml of KRBB containing 50 units of heparin per milliliter. At the end of incubation (2 min; 37 °C) and centrifugation, the supernatant was tested for vasorelaxant activity as described, except that a concentration-response relationship was determined in a cumulative fashion. Control experiments were conducted by incubating the platelets in KRBB alone. Heparin (50 U/ml) was added to the supernatant of the control mixture after incubation and removal of the platelets by centrifugation to control for the effect of heparin.
Figure 5. Relaxation of contracted rabbit aortic rings by rat high molecular weight atrial natriuretic peptides (HMWP) that had been incubated with rat platelets (H-platelets) or with rat platelet-free plasma (H-platelets + heparin). Conditions as in Figure 3. In the experiments in which samples were incubated without heparin, heparin was added to the HMWP after removal of the platelets by centrifugation to control for the effects of heparin on relaxation of aorta. Means ± se are shown (n = 6 for each point for H-platelets, and n = 5 for each point for H-platelets + heparin); *p < 0.02.
alone. Thus, as with heparinized blood, the HMWP incubated in plasma probably underwent some molecular weight change, but certainly not to the degree that had occurred when HMWP were incubated in whole blood. Thus, since plasma incubation produced very little natriuretic activity in the less than 10,000-dalton range we contend that the plasma was not responsible for the major change in the molecular weight of HMWP that occurred in whole blood.

In contrast, when the HMWP were incubated with rat platelets, natriuretic activity eluted almost exclusively in the less than 10,000-dalton range. Furthermore, the molecular weight change was similar to that which occurred when the HMWP were incubated in rat blood. These results suggest that the platelets were predominantly responsible for the conversion of HMWP to LMWP that took place in whole blood. This converting activity of platelets was destroyed by heat, which suggests that it was due to a substance that required a tertiary structure to be active. The platelet converting factor may also have required the platelets to be intact in order for it to be active. Further, it was found that the presence of heparin in the incubation medium inhibited the platelet-associated converting activity, which was similar to the heparin inhibition of converting activity observed in whole blood.

There was a certain degree of variability in peak sizes of natriuretic activity among the various experiments. Although part of this variability probably was due to variations in the bioassay and in recoveries from gel columns, we contend that other sources of variations were more important. When HMWP were incubated with the blood or blood components, at least four processes could have influenced the level of natriuretic activity and the subsequent peak sizes. A certain process could have influenced the level of natriuretic activity, which was similar to the heparin inhibition of converting activity observed in whole blood.

To determine if conversion of HMWP to LMWP was associated with enhancement of biological activity, we compared the vasorelaxant activity of KRBB-treated HMWP with that of platelet-treated HMWP. The isolated aortic ring preparation was chosen to measure biological activity because possible in vivo conversion and activation might have complicated interpretation of the results had we used the intact rat for natriuretic activity. We found that the platelet-treated HMWP had significantly greater aortic relaxant activity as compared with that of KRBB-treated HMWP. In contrast, when the LMWP was examined under these same conditions, platelet incubation had no effect on the vasorelaxant activity. The ability of platelets to enhance the vasorelaxant activity of HMWP was inhibited by the presence of heparin in the incubation medium (Figure 5). This observation concurs with the finding that heparin inhibited the ability of both whole blood and platelets to convert HMWP to LMWP. These results are in concert with the hypotheses that incubation of HMWP with rat platelets in vitro causes HMWP to be proteolytically converted to LMWP, which in turn are more biologically active, and that LMWP are not activated by proteolysis.

In vitro conversion and activation of rat HMWP by proteolysis has been demonstrated by others, which suggests that in vivo processing of the rANP precursor might be required for full expression of biological activity. However, it is not clear whether the rANP precursor is processed within the atrial myocytes to form active LMWP or if pro-ANP is released and processed extracellularly or whether a combination of both occurs. The observation that rat atrial extracts contain a heat-labile factor capable of converting HMWP to LMWP supports the notion that conversion and activation take place within the myocyte or during release. This idea is also supported by the study of Currie et al. Using a bioassay to follow ANP activity, they reported that only a low molecular weight atrial peptide was released from the isolated perfused rabbit heart. However, there is also evidence that pro-ANP may be present in the circulation. Using high-performance liquid chromatography and an ANP radioimmunoassay, Gutkowska et al. reported the presence of a small peak of immunoreactive ANP in rat plasma that did not elute in the same vicinity as that of a low molecular weight ANP standard. It is not uncommon in peptide hormone processing for propeptide to be released in small quantities along with other products. For example, during the secretion of insulin from the pancreatic B cell, approximately 3 to 4% of the released material is in the form of proinsulin. If this proves to be the case for pro-ANP, the results of the present study suggest that, in rats, circulating platelets might participate in converting this precursor to the more active low molecular weight forms.

More work is needed to characterize the platelet-associated converting factor for rANP in order to assess its biological importance. For instance, studies will be needed to determine 1) whether platelet conversion of HMWP occurs in vivo, 2) its time course of action, 3) the minimum concentration of platelets needed for conversion, 4) whether the converting factor is located internally or on the cell membrane, 5) whether it requires the presence of divalent ions such as calcium, 6) whether it can be inhibited by characteristic inhibitors such as aprotinin, 7) whether or not it is associated with platelets of species other than the rat, and 8) whether or not the platelet converting activity is a selective enzyme event.
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