Are cardiovascular and sympathoadrenal effects of human "new pressor protein" preparations attributable to human coagulation β-FXIIa?

Peter C. Papageorgiou,[1,2,3]* Ali Pourdjabbar,[1,3]* Akis A. Amfilochiadis,[1,2,3] Eleftherios P. Diamandis,[4] Frans Boomsma,[5] and Daniel H. Osmond[1,2]

[1]*Departments of Physiology and Medicine, University of Toronto, Toronto, Ontario M5S1A8;* [2]*Heart and Stroke/Richard Lewar Centre of Excellence, University of Toronto, Toronto, Ontario M5S3E2;* [3]*Institute of Medical Science, University of Toronto, Toronto, Ontario M5S1A8;* [4]*Department of Laboratory Medicine and Pathobiology, University of Toronto and Mount Sinai Hospital, Toronto, Ontario, Canada M5G1X5;* [5]*Department of Internal Medicine, Erasmus Medical Centre, Rotterdam 3015 GD, The Netherlands*

Papageorgiou, Peter C., Ali Pourdjabbar, Akis A. Amfilochiadis, Eleftherios P. Diamandis, Frans Boomsma, and Daniel H. Osmond. Are cardiovascular and sympathoadrenal effects of human "new pressor protein" preparations attributable to human coagulation β-FXIIa? *Am J Physiol Heart Circ Physiol* 286: H837–H846, 2004. First published October 23, 2003; 10.1152/ajpheart.00693.2003.— "New pressor protein" (NPP) derived from normal human plasma is an extra renal enzyme that shares strong sequence homology with human coagulation β-FXIIa. Under our bioassay conditions, human NPP (10–20 μl plasma equivalent/~300 g rat iv) can raise the systolic blood pressure (SBP) by 40–50 mmHg, the diastolic blood pressure (DBP) by 15–20 mmHg, and the heart rate (HR) by 70–90 beats/min. Plasma epinephrine (of adrenal medullary origin) and norepinephrine rise by about 50- and 10-fold, respectively. Because β-FXIIa is not normally associated with pressor properties, we endeavored to substantiate that the hypertensive effects of impure NPP preparations used in our experiments are attributable to their content of β-FXIIa. We carried out comparisons with highly purified (>90%) commercial human β-FXIIa and found that by gel filtration (Sephadex G-100 and G-75), NPP bioactivity appeared in the ~30-kDa elution zone, consistent with the molecular mass of β-FXIIa. Retention time using fast-protein liquid chromatography anion exchange chromatography was identical. Molecular mass and comigration were confirmed by SDS-PAGE gel electrophoresis, and the recovered ~30-kDa protein bands yielded β-FXIIa fragments identified by mass spectrometry. Matched doses of the NPP preparations produced dose-response curves very similar to those elicited by β-FXIIa with respect to increments of SBP, DBP, and HR, whereas plasma catecholamine increments were generally comparable. We propose that β-FXIIa is substantially, if not exclusively, responsible for the observed effects of our NPP preparations and that this points to a novel axis connecting the FXII coagulation cascade and the sympathoadrenal gland to other cardiovascular regulatory mechanisms.

Hageman fragment factor; hypertension; catecholamines

WE HAVE REPORTED on the properties of an extrarenal, enzymic protein derived from normal human and rat plasmas tentatively called "new pressor protein" (NPP) (18, 19, 21, 25). This designation was used originally because the effects of NPP could not be attributed to any identifiable pressor agent, including renin-angiotensin and endothelin, on the basis of structural or functional characteristics.

Physiologically, miniscule bolus injections of impure human or rat NPP (10–20 μl plasma equivalent/~300g rat iv) produced biphasic or multiphasic blood pressure (BP) responses, notably a small, brief initial depressor phase followed by a much more prominent, prolonged pressor phase lasting 10–15 min, together with a marked elevation of heart rate (HR) (19). In our ganglion-blocked (pentolinium) bioassay, rats pretreated with the angiotensin-converting enzyme inhibitor captopril (which potentiates the effects), such small doses of human NPP raised systolic BP (SBP) by 40–50 mmHg, diastolic BP (DBP) by 15–20 mmHg, and HR by 70–90 beats/min. The cardiac stroke volume was also elevated such that cardiac output was significantly increased without a real change in total peripheral resistance, suggesting that NPP acts primarily on the heart via chronotropic and cardiotonic mechanisms (19). Recently, we reported the presence of NPP in anephric hemodialysis patients as a possible contributing factor to their hypertension (22).

Concurrent with these potent cardiovascular effects, there was a massive elevation of plasma catecholamine levels clearly demonstrated to be predominantly of adrenal medullary origin (19, 21). Plasma epinephrine levels increased 50-fold, or more, whereas norepinephrine levels increased about 10-fold, greatly elevating the epinephrine-to-norepinephrine ratio to 20:1, whereas increases in dopamine were much more modest (19, 25). Such high levels of plasma catecholamines suggested that they were involved in the mechanism of action of NPP and contributed to its observed cardiovascular effects. Current evidence suggests that NPP generates, and/or recruits, a peptide(s) that triggers the release of adrenal medullary catecholamines to elicit the observed effects (19, 25). Such a "cooperative" mode of action, initiated by NPP, proposes the existence of an axis linking coagulation FXII to sympathoadrenal and cardiovascular regulatory mechanisms, which hypothesis is under active investigation.

Biochemical studies of human NPP revealed that it was a heat labile enzymatic protein with a molecular mass of ~30 kDa and an isoelectric point of 4.7–4.9 (21). Its NH2-terminal sequence (19 amino acid residues) indicated strong homology with the heavy chain of the β-fragment of activated human coagulation factor XII (β-FXIIa) (21). Supporting this structural relationship with human FXII was evidence that of all the congenitally deficiency plasmas tested (factors XI, XII, prekal-

*P. C. Papageorgiou and A. Pourdjabbar contributed equally to this study.

Address for reprint requests and other correspondence: D. H. Osmond, Dept. of Physiology and Medicine, Faculty of Medicine, Univ. of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8 (E-mail: daniel.osmond@utoronto.ca).

likrein, and kininogen) only severely FXII-deficient plasmas failed to generate significant NPP activity (18). Such activity could be restored by adding suitable amounts of highly purified human coagulation FXII or its derivatives followed by controlled activation with trypsin (18). Direct intravenous injection of a highly purified preparation of β-FXIIa elicited NPP-like pressor responses (18). Moreover, the corn trypsin inhibitor of FXII (12) added in vitro was able to inhibit pressor activity of NPP in vivo (21).

Such a proposed relationship between NPP and coagulation β-FXIIa attributes novel, unexpected, physiological properties to the FXII cascade that are worthy of careful verification, one requirement being the availability of suitably pure preparations of NPP. All our initial observations were unavoidably based on impure preparations of NPP obtained from both human and rat plasmas, which appeared to be equipotent in our bioassay rats (21). Human NPP produced remarkably reproducible results in bioassay rats, especially when prepared from the same plasma pool or from matched pools (19). Such impure preparations of both human and rat NPP were used in our laboratory to maintain continuity between related series of experiments, to accumulate sufficient physiological data to establish the proposed NPP paradigm, and to evaluate its significance. It was possible and necessary to do this even before sequence identity of NPP was determined or adequate quantities of highly purified material were available for ongoing animal experiments. Anything short of complete purification of NPP would not have resolved questions as to the contribution of any contaminants remaining in the preparation being investigated.

Accordingly, the aim of this report is *1*) to describe a reasonably simple method for obtaining sizeable quantities of partially purified NPP (ppNPP) from normal human plasma, as well as more limited quantities of a more highly purified derivative (FPLC-NPP, prepared from ppNPP); *2*) to characterize the properties of ppNPP preparations in relation to impure NPP (NPP); *3*) to characterize a highly purified commercial preparation of the β-fragment of activated human coagulation factor XII (β-FXIIa); *4*) to compare the relevant biochemical and physiological characteristics of these NPP, ppNPP, and β-FXIIa preparations; and *5*) to extend our knowledge of the physiological actions of coagulation β-FXIIa.

Improved knowledge of the functions of NPP relative to β-FXIIa is worthwhile because human coagulation FXII can be readily activated in the body as a result of inflammatory, thrombotic, traumatic, and related conditions (6). As a derivative of such endogenous activation, β-FXIIa could become available in the circulation to exert effects similar to those produced by exogenous β-FXIIa. The fact that the actions of NPP are potentiated in bioassay animals treated with the angiotensin-converting enzyme inhibitor captopril (21) makes it all the more relevant to clarify its relationship to human coagulation β-FXIIa for clinical (22) as well as physiological reasons.

MATERIALS AND METHODS

Human Plasma

Human plasmas, considered to be normal but unsuitable for human transfusion, were provided by the Canadian Blood Services (formerly the Canadian Red Cross Society, Toronto Centre) and handled as described previously (18, 21).

Preparation of Human NPP

Controlled tryptic activation of human plasma was carried out as described previously (18, 21). Briefly, trypsin (type III, bovine, T-8253; Sigma-Aldrich) was prepared as a stock solution in 0.002 mol/l HCl and added to plasma, with mixing, at 3–10% vol/vol to achieve a final trypsin concentration of 1 mg/ml, ensuring minimal plasma dilution. After incubation for 10 min at 23°C, the reaction was terminated by adding excess lima bean trypsin inhibitor (LBTI, T-9378, type II-L, Sigma-Aldrich) dissolved in 0.1 mol/l Tris-acetate buffer (45 mg/ml, pH 7.4), usually 1.2 mg LBTI/mg of added trypsin. The sample was divided into 100-μl aliquots and kept frozen at −40°C until time of use. Each experimental day, a fresh tube of this activated plasma preparation (designated NPP) was thawed, kept on ice, and used for bolus intravenous injections into bioassay rats in doses expressed as human plasma equivalent or as protein content in this preparation per kilogram rat body weight (mg/kg). For continuity with previous experiments, it was calculated that human NPP at 20 μl plasma equivalent per rat is equivalent to human NPP at a dose of 5.5 mg protein/kg.

Preparation of Partially Purified NPP by Size Exclusion Chromatography

Human plasma (10 ml), activated with trypsin as described above for the preparation of NPP, was filtered through Whatman no. 1 filter paper (Whatman International; Kent, UK) and applied to a calibrated Sephadex G-100 (regular) gel filtration column [100 cm × 2.6 cm inner diameter (ID), Pharmacia]. The sample was eluted with physiological saline (0.9% NaCl in distilled water) at a constant rate of 0.3 ml/min using a peristaltic pump P3 (Pharmacia). All steps were carried out in a cold room at 4°C. The optical density (OD) of each 3-ml fraction was determined at 280 nm using a Beckman DU-64 Spectrophotometer (Beckman Instruments; Fullerton, CA). Aliquots (100 μl) of each eluted fraction were tested for activity using bioassay rats (see *Rat Bioassay: Direct BP and HR Recordings*), and active fractions were pooled, lyophilized, and desalted on a Sephadex G-25 (fine) gel filtration column (30 cm × 1 cm ID, Pharmacia) and lyophilized again. Molecular mass was estimated by calibrating the column using the molecular markers albumin (64 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12 kDa).

The desalted and lyophilized powder representing active fractions from the Sephadex G-100 columns was again reconstituted in distilled water (to 5 ml), filtered through Whatman no. 1 filter paper, and applied to a calibrated Sephadex G-75 gel filtration column (100 cm × 2.6 cm ID, Pharmacia). The sample was eluted as described above. Fractions (3 ml) were collected, their OD measured, and their activity tested in bioassay rats. The active fractions were pooled, lyophilized, desalted, and lyophilized again, as described above, and stored for future use at −40°C. Calibration of the column was with albumin (64 kDa molecular mass) and carbonic anhydrase (30 kDa). Human NPP at this stage of purity is designated as partially purified NPP (ppNPP).

Human Coagulation β-FXIIa Preparation

Highly purified coagulation β-FXIIa (30 kDa, Lot β-FXIIa 1000P) was obtained from Enzyme Research Laboratories (South Bend, IN). It had been prepared from pure (>95%) human Factor XII (80 kDa, HFXII 1212, Enzyme Research Laboratories) and was received as a frozen solution on dry ice. The concentration was given as 1.03 mg/ml dissolved in 4 mM sodium acetate HCl-0.15 M NaCl buffer (pH 5.3). This preparation was stated to be of higher purity than the preparation we reported previously from the same source (18), in this case >90% β-FXIIa (30 kDa) with a <10% impurity of α-FXIIa (50 kDa). Aliquots (50 μl) of this stock solution were made up to 500 μl in 0.9% NaCl to a concentration of 100 ng/μl. Aliquots (5 μl) of this second stock solution were made up to 50 μl in 0.9% NaCl to a concentration

of 10 ng/μl, which was used for bolus intravenous injections into bioassay rats at the specified doses. All solutions of coagulation β-FXIIa were stored at −40°C until the time of use and kept on ice during experiments.

Rat Bioassay: Direct BP and HR Recordings

All animals were cared for and used in accordance with the principles and guidelines outlined by the Canadian Council on Animal Care and all experimental protocols were approved by the relevant Animal Care Committees of the Faculty of Medicine and the University of Toronto. Male Wistar rats, weighing ~250–300 g (Canadian Biobreeding Laboratories, Charles River) were prepared for bioassay according to Pickens et al. (23), with modifications, as described by Osmond et al. (21) and further below. The rats were housed in standard metal cages with free access to regular Purina rodent laboratory chow (Ralston Purina; St. Louis, MO) and tap water.

The rats were anesthetized with Inactin (sodium ethyl-[1-methyl-propyl]malonyl-thio-urea, 100 mg/kg ip, Promonta; Hamburg, Germany) and given atropine sulfate (2.4 mg/kg sc, DIN 153656, Ormond Veterinary Supply; Toronto, Canada) to reduce any cardiac depression from the anesthetic and bronchial secretions during surgery. Through a midline incision, a tracheostomy was performed just below the isthmus of the thyroid to secure a patent airway, and the vagus nerve trunks on both carotid arteries were severed at the same level to block reflex bradycardia. A common carotid artery was cannulated using PE-50 polyethylene tubing (Clay Adams, Becton Dickinson) for arterial SBP-DBP and HR recordings, using Statham DC pressure transducers (Hato Rey) connected to a MacLab/8 data acquisition system (AD Instruments and Lamont Scientific, Toronto, Canada) connected to a Power Macintosh 7200/1200 PC compatible computer and driven by MacLab Chart version 3.5.6 software. The carotid artery cannula was filled with heparinized saline (physiological saline containing 20 U/ml of heparin; Hepalean, Organon Teknika, Ontario, Canada) and occasionally flushed (~0.1 ml) to prevent coagulation. A mercury sphygmomanometer (Tycos, Taylor Instruments) was used to calibrate the recording system every experimental day.

All injections were made via a 27-gauge needle attached to a PE-20 polyethylene cannula (Clay Adams, Becton Dickinson), which was inserted freely into a femoral vein, without ligation, to allow venous return at the injection site. Injections were flushed in with up to 0.1 ml of physiological saline. The ganglion-blocking agent Ansolysen (pentolinium tartrate salt, P-3520, Sigma-Aldrich) was administered at 19.2 mg/kg dissolved in polyvinylpyrrolidone (PVP-40T, Sigma-Aldrich), allowing for its gradual release during the experimental period. The angiotensin-converting enzyme inhibitor captopril (C-4042, Sigma-Aldrich) was dissolved in physiological saline and administered intravenously at 2.5 mg/kg in two injections 20 min apart starting >40 min before bioassay. Only rats that had sustained minimal tissue trauma and blood loss during surgery and had stable BP (~80/40 mmHg) and HR (~350 beats/min) at the outset were used.

Biochemical Characterization of Human NPP and Coagulation β-FXIIa

FPLC anion exchange chromatography. ppNPP was reconstituted in distilled water to a final protein concentration of 1 μg/μl, and 350 μg were loaded onto a Polyanion SI HR5/5 anion exchange column (no. 17-0549-01, Pharmacia) attached to a dual pump FPLC chromatography system (Pharmacia). The NPP was eluted with *buffer A* (0.015 M Tris·HCl, and 0.075 M NaCl, pH 8.0) and *buffer B* (0.015 M Tris·HCl, and 0.65 M NaCl, pH 8.0) at a flow rate of 0.5 ml/min. The programmed gradient conditions were the following: 0–10 min at 0% *buffer B*; 10–50 min linear gradient from 0 to 100% *buffer B*; 50–75 min at 100% *buffer B*; 75–80 min at linear gradient from 100 to 0% *buffer B*; and a washout period of 80–85 min at 0% *buffer B*. The OD of each 0.5-ml fraction was measured at 280 nm (Beckman

DU-64, Beckman Instruments; Fullerton, CA), and 50-μl aliquots of each fraction were tested for NPP activity using bioassay rats. The active fractions were pooled, lyophilized, desalted, and lyophilized again, as described above, and stored at −40°C. Human NPP purified using FPLC anion exchange at this stage of purity is designated as FPLC-NPP. The identical procedure was also executed loading 50 μg of human coagulation β-FXIIa onto the anion exchange column.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (14) in gels formed from 14% and 17.5% separating acrylamide gels. Each well was loaded with ppNPP, FPLC-NPP, or commercial coagulation β-FXIIa, with a specified microgram quantity of protein between tracks loaded with low-molecular-weight markers (Pharmacia) and electrophoresis carried out (Bio-Rad, Mini Protean II Cell) at 30 mA constant current per gel, using 25 mM Tris·HCl and 192 mM glycine (pH 8.3), 22°C, until the dye front reached the bottom of the gel. The gel was then stained with 0.125% Coomassie brilliant blue in 50% methanol + 10% acetic acid and destained using 10% methanol + 5% acetic acid. The gels were later photographed (Photography Centre, Medical Sciences Building, Faculty of Medicine, University of Toronto).

Protein identification using mass spectrometry. After separation by FPLC-anion exchange chromatography and SDS-PAGE, the recovered protein bands (from Fig. 5) were isolated and subjected to proteolytic digestion with trypsin, as previously described (15, 24). Positive identification of protein fragments was determined by using nanoelectrospray tandem mass spectrometry.

Physiological Characterization of Human NPP and Coagulation β-FXIIa

Cardiovascular effects of human NPP, ppNPP, and coagulation β-FXIIa. Dose-response experiments using human NPP, ppNPP, and coagulation β-FXIIa were conducted using bioassay rats. To avoid tachyphylaxis (due to multiple injections), the bioassay rats were divided into three groups based on dose to limit the number of injections received by each animal. The peak SBP, DBP, and HR were recorded after each injected dose, and sufficient time was allowed to elapse (~15 min) between successive injections to allow a return to baseline BP and HR values before the next injection.

HUMAN NPP. One group of rats received human NPP (stock) incrementally at 0.05, 0.1, 0.2, and 0.5 μl iv of plasma equivalent. This represents 0.01, 0.02, 0.04, and 0.1 mg/kg of protein, respectively. The NPP stock was diluted 100-fold with physiological saline to enable volumes of at least 5 μl to be injected with greater accuracy than is possible with smaller volumes. A second group received human NPP intravenously at 0.5, 1, 1.5, 2, and 5 μl of plasma equivalent, which represents 0.1, 0.2, 0.3, 0.6, and 1.4 mg/kg of protein. The NPP in this group was diluted 10-fold with physiological saline to facilitate more accurate injections. The third group received undiluted human NPP intravenously and incrementally at 5, 10, and 20 μl of plasma equivalent, which represents 1.4, 2.8, and 5.5 mg/kg of protein, respectively.

PPNPP. ppNPP was reconstituted in physiological saline to a final concentration of 0.1 and 1 μg/μl. The first group of rats received ppNPP injections at 1, 5, 10, and 15 μg/kg (at 0.1 μg/μl). The second group received ppNPP at 15, 25, and 35 μg/kg (at 1 μg/μl). The third group received ppNPP at 35, 50, and 100 μg/kg (at 1 μg/μl).

COAGULATION B-FXIIA. Highly purified coagulation β-FXIIa was reconstituted in physiological saline to a final concentration of 1 and 10 ng/μl. The first group of bioassay rats received β-FXIIa at 15, 30, 80, and 160 ng/kg (using stock at 1 ng/μl). The second group received β-FXIIa at 160, 200, and 300 ng/kg (using stock at 10 ng/μl). The third group received β-FXIIa at 300, 600, and 1,000 ng/kg (using stock at 10 ng/μl).

Determinations of plasma catecholamines in response to NPP, ppNPP, and coagulation β-FXIIa. Three arterial blood samples (1 ml each) were withdrawn via the carotid artery after taking care to

displace all the saline by allowing blood to rise in the cannula. The first (baseline) sample was taken 10 min before the injection of human NPP, ppNPP, or coagulation β-FXIIa. The second (peak) sample was drawn at ∼2 min into the pressor response, corresponding to the maximum increase in SBP pressure, and the third (recovery) sample was drawn at 20 min after injection when the BP and HR had returned to baseline. Blood (1 ml) from a donor rat was infused into the bioassay rat ∼10–15 min before withdrawal of each blood sample to avoid hypovolemia at the time of blood sampling. These donor rats, weighing 350–450 g, were prepared in the same way as the bioassay rats, and their extracellular volume was maintained by injecting 1 ml of physiological saline via the femoral vein 10 min before removal of each blood sample.

The blood collected was immediately transferred to a chilled polystyrene tube containing 1.2 mg glutathione (Boehringer Mannheim) in 500 μl heparinized saline (10 U/ml, Hepalean) to retard catecholamine oxidation and blood coagulation, respectively, and then stored on ice. The syringe and needle used to collect the blood were rinsed (∼100 μl) with the heparinized saline-glutathione solution to guard against catecholamine oxidation and blood coagulation during the blood sampling.

The collected blood samples were centrifuged at 2,000 g for 20 min at 4°C, and the plasma was separated, placed into chilled polystyrene tubes in 300-μl aliquots, and stored at −40°C until the time of assay. Plasma catecholamine levels of epinephrine, norepinephrine, and dopamine were determined by using high-performance liquid chromatography with fluorimetric detection (29). This fluorimetric method is sensitive enough to deal with small plasma volumes (∼250 μl) and detect picogram levels of plasma catecholamines.

Statistical Analyses

Numerical data were analyzed using the Sigma Stat program (version 2.03, SPSS; San Rafael, CA). SBP, DBP, HR, and plasma catecholamine data are presented as means ± SE. All changes in these values as a result of the various injections (ΔSBP, ΔDBP, and ΔHR) were calculated from baseline and statistically compared with corresponding values using the unpaired one-tailed distribution Student's t-test with probability values indicated. Statistical comparisons of plasma catecholamines within the treatment group were analyzed by using the one-way repeated measures analysis of variance tests using the Bonferroni test for multiple comparisons, and between treatment groups were analyzed by using the unpaired one-tailed distribution Student's t-test, with probability values indicated. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Purification of Human NPP by Size Exclusion Chromatography

Sephadex G-100. Major elution of plasma proteins began at fraction 43 and continued until about fraction 88, with little differentiation into specific peaks (Fig. 1A). NPP activity (ΔSBP, mmHg) was detected in fractions 63–95, with peak activity found in fraction 82. This activity peak corresponded to an estimated molecular mass of 30 kDa, according to the calibration marker protein carbonic anhydrase.

Sephadex G-75. The lyophilized product of pooled active fractions from the Sephadex G-100 gel (see above) was further fractionated using a Sephadex G-75 column. A sharper protein peak was obtained (fractions 55–78) with the apex at about fraction 67 (Fig. 1B). The NPP activity was found to the right of the protein peak between fractions 72 and 100 with its apex in fraction 86, corresponding to an estimated molecular mass of 30 kDa.
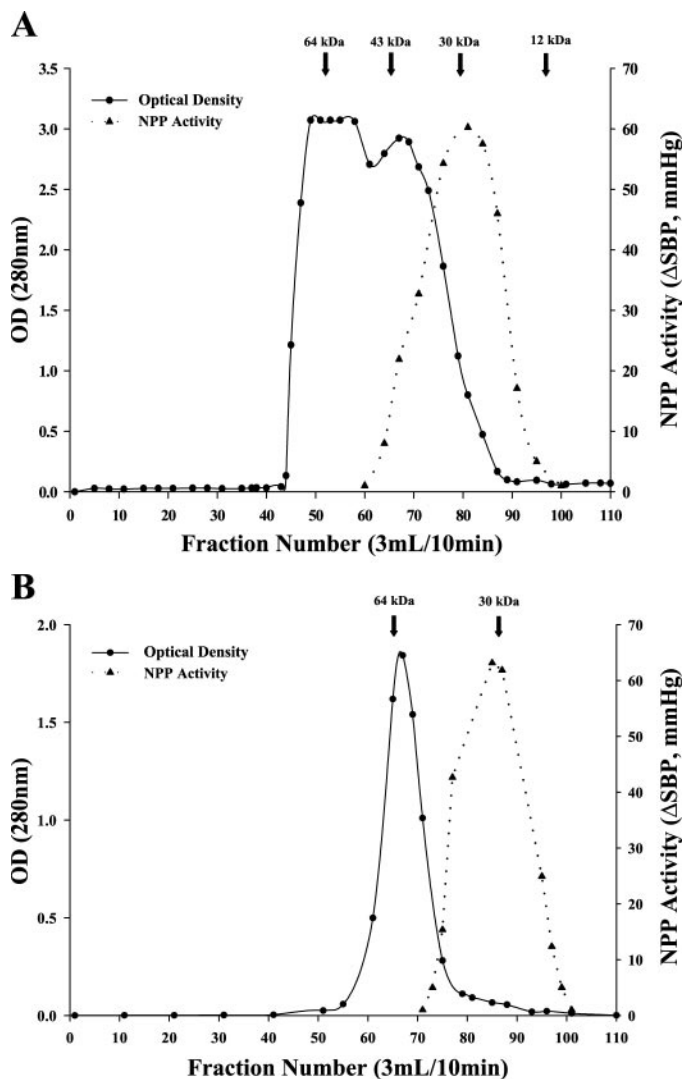

Fig. 1. Human new pressor protein (NPP) Sephadex G-100 and G-75 elution profiles. The Sephadex G-100 elution profile (*A*) of trypsin-activated human plasma NPP and the zone of bioactivity eluted through a Sephadex G-75 column (*B*). Protein content (optical density, OD) and NPP activity of eluted fractions (see MATERIALS AND METHODS). Elution pattern is typical of several runs. NPP obtained after both Sephadex G-100 and G-75 purification is designated partially purified NPP (ppNPP).

Gel Electrophoresis of Coagulation β-FXIIa and Partially Purified NPP

Duplicates of highly purified commercial βFXIIa (5 and 10 μg protein) and ppNPP (20 and 30 μg protein) were loaded onto a SDS-PAGE gel (14% acrylamide) between tracks loaded with low-molecular-weight markers. The β-FXIIa separated into a major band at ∼30 kDa, and the ppNPP separated into a large band at ∼67 kDa with several faint bands between 35 and 67 kDa (Fig. 2).

Purification by FPLC Anion Exchange Chromatography

ppNPP. A ppNPP preparation (350 μg of protein) was loaded onto a Polyanion SI HR5/5 anion exchange column attached to a FPLC chromatography system and eluted as described (MATERIALS AND METHODS, Fig. 3). Several well-
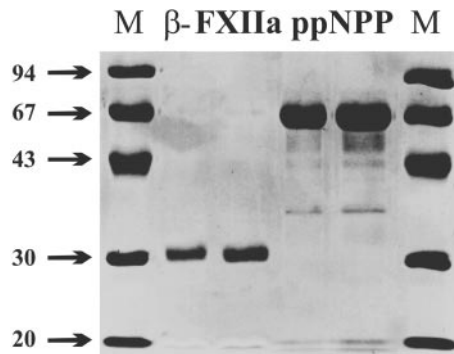
Fig. 2. SDS-PAGE electrophoresis of commercial coagulation β-FXIIa and ppNPP. SDS-PAGE (14% acrylamide gel) of highly purified human coagulation β-FXIIa (5 and 10 μg protein load/track) and partially purified NPP (ppNPP, 20 and 30 μg protein load/track) relative to standard molecular markers (M, molecular mass in kDa, MATERIALS AND METHODS).

dispersed protein peaks of different heights were detected. The collected fractions were injected (50 μl) into bioassay rats and tested for NPP activity, which was found only in fractions 33–42. The peak NPP activity was found in fraction 37 (ΔSBP of 52 mmHg), which corresponds to a major protein peak.

β-FXIIa. Highly purified β-FXIIa (50 μg of protein) was fractionated using the same chromatography system as described above. Only one major protein peak was detected (Fig. 4). Subsequently, the collected fractions were tested for NPP activity (as above), which was found only in fractions 32–42. The peak of NPP activity (fraction 38, ΔSBP of 53 mmHg) corresponded to the major protein peak.

Gel Electrophoresis of FPLC-NPP and Coagulation βFXIIa

Duplicates of FPLC-NPP (30 μg protein) and highly purified human β-FXIIa (20 μg protein) were loaded onto a SDS-PAGE gel (17.5% acrylamide) between tracks loaded with low-molecular-weight markers. The FPLC-NPP separated into a heavy band at ~67 kDa with well-defined bands at ~18 and 30 kDa (Fig. 5). The β-FXIIa separated into a major defined band at ~30 kDa with a small band between 43 and 67 kDa.

Protein Identification by Mass Spectrometry

We identified the protein bands from the gel electrophoresis of FPLC-NPP and coagulation β-FXIIa (from Fig. 5) by using nanoelectrospray tandem mass spectrometry. The top band from the FPLC-NPP lane, which migrated to ~67 kDa, gen-erated fragments identified as LVNEVTEFAK, AFKAWA-VAR, LSQRFPKAEFAEVSK, RHPDYSVVLLLR, and VF-DEFKPLVEEPQNLIK. These fragments correspond to amino acids 66–75, 234–242, 243–257, 361–372, and 397–413 of human serum albumin (*Homo Sapiens*, Genebank Accession no. AAN17825), respectively. The middle band from the FPLC-NPP lane, which migrated at ~30 kDa, generated fragments identified as VVGGLVALR and LHEAFSPVSYQHD-LALLR. These fragments correspond to amino acids 373–381 and 449–466, respectively, of human coagulation FXII (Hageman factor, *Homo Sapiens*, GenBank Accession no. NP_000496), specifically to the heavy chain of β-FXIIa. The lower band from the β-FXIIa lane, which migrated at ~30 kDa, generated fragments identified as VVGGLVALR, GGLVALR, PAPEDLTVVLGQER, and LHEAFSPVSYQH-DLALLR. These fragments correspond to amino acids 373–381, 375–381, 418–431, and 449–466, respectively, of human coagulation FXII (Hageman factor, *Homo Sapiens*, GenBank Accession no. NP_000496), specifically to the heavy chain of β-FXIIa. The protein content of the bottom band (18 kDa) from the FPLC-NPP lane and the top band (between 43 and 67 kDa) from the β-FXIIa lane was insufficient for analysis by mass spectrometry.

Cardiovascular Effects of Human NPP, ppNPP, and Coagulation βFXIIa

The general profile of the dose-response curves and the magnitude of SBP, DBP, and HR responses relative to increasing doses of the various preparations are all sufficiently comparable to suggest that all effects were produced by a common agent they contained, as elaborated below.

Human NPP dose-response curves. Human NPP raised the SBP and the DBP in a dose-dependent manner reaching a plateau at doses 1.4 and 0.45 mg/kg, respectively, with the SBP increment at about 50 mmHg and the DBP at about 20 mmHg (Fig. 6A). At the higher dose the HR increased by about 80 beats/min further increasing to about 90 beats/min with doses of 2.8 mg/kg or higher (Fig. 6B). For purposes of continuity with past experiments, a dose of 5.5 mg/kg (or 20 μl plasma equivalent) was used as the standard dose at this level of purity, which raised SBP by 51 ± 2 mmHg, DBP by 24 ± 2 mmHg, and HR by 89 ± 3 beats/min.

ppNPP dose-response curves. The dose-dependent manner in which human ppNPP elevated the SBP, DBP, and HR was comparable to the pattern given by human NPP. The SBP
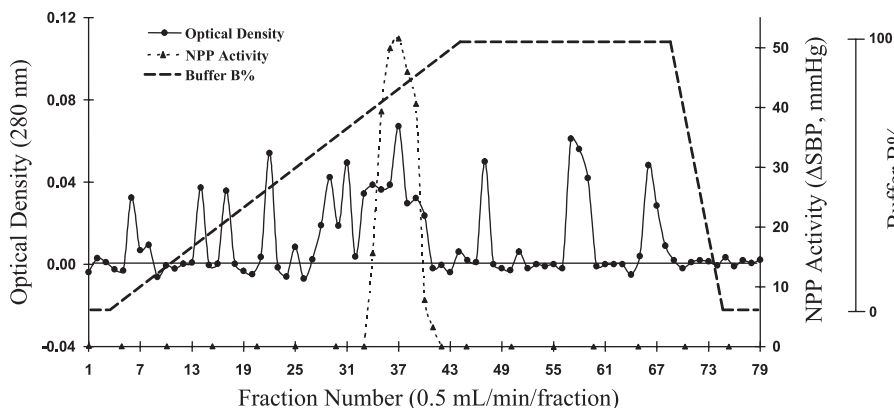

Fig. 3. FPLC anion exchange chromatography of ppNPP. Elution profile of ppNPP (350 μg protein load) using a two-buffer gradient. Slope (dashed line) represents %*buffer B* (MATERIALS AND METHODS). Protein content (solid line) of eluted fractions was determined at 280 nm. Eluted fractions were tested for NPP activity [change in systolic blood pressure (ΔSBP), mmHg] by injecting 50 μl iv of each fraction into bioassay rats (dotted line, triangles). Every 5th fraction between 1 and 79 was tested until activity was found, at which point every fraction was tested individually. Pressor activity was found only in fractions 33–42. This elution pattern is typical of several runs.
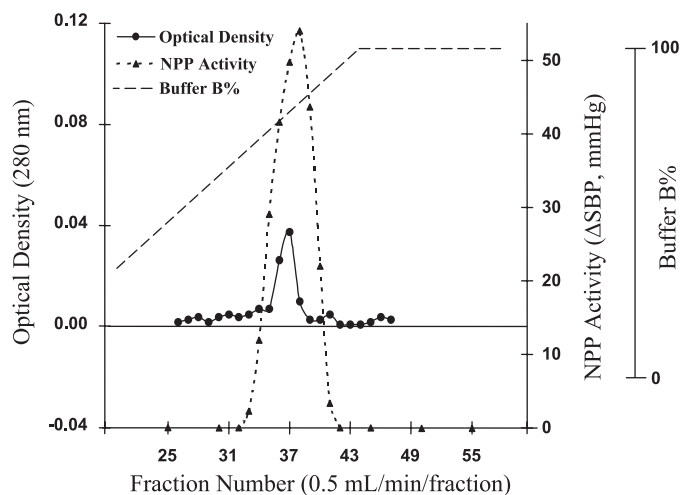
Fig. 4. FPLC anion exchange chromatography of commercial human coagulation β-FXIIa. Human coagulation β-FXIIa (50 µg protein) was eluted by FPLC anion exchange (MATERIALS AND METHODS). Slope (dashed line) represents %*buffer B* (MATERIALS AND METHODS). Protein content (solid line) was determined in terms of OD at 280 nm. Eluted fractions were tested for NPP activity (ΔSBP, mmHg) by injecting 50 µl iv of each fraction into bioassay rats (dotted line, triangles). Every 5th fraction (fractions 1–79) was tested until activity was found, at which point all fractions were tested individually. Pressor activity for β-FXIIa was found only in fractions 32–42. This elution pattern is typical of several runs.

reached a plateau at ~55 mmHg by using a 50 µg/kg dose of ppNPP, and the DBP and HR plateaued at ~20 mmHg and 75 beats/min, respectively, by using a dose of 35 µg/kg (Fig. 6, *C* and *D*). At this level of purity, a dose of 35 µg/kg, which increased SBP by 47 ± 3 mmHg, DBP by 22 ± 2 mmHg, and HR by 74 ± 7 beats/min, was used as the standard dose for purposes of comparison with other preparations.

Highly purified coagulation β-FXIIa dose-response curves. Commercially obtained highly purified human coagulation β-FXIIa increased SBP, DBP, and HR very similarly to the pattern seen with human NPP and ppNPP. The responses started to plateau at a dose of 300 ng/kg, without significant increments thereafter (Fig. 6, *E* and *F*). At 300 ng/kg the peak SBP increments reached 45 ± 6 mmHg, the DBP peak increment was 22 ± 5 mmHg, and the HR peak increment was 70 ± 10 beats/min.

Comparison of Cardiovascular Effects of Human NPP, ppNPP, and Coagulation β-FXIIa

Degree of purification of compared preparations. As previously stated, the standard dose of human plasma NPP administered to bioassay rats in most previous experiments was generally 20 µl plasma equivalent per ~300 g rat (iv). This volume was calculated to contain 1.65 mg of protein, corresponding to a 5.5 mg/kg dose for a ~300-g bioassay rat. The SBP increase of about 45–50 mmHg produced by this dose was fairly closely matched by 35 µg/kg of ppNPP and by 300 ng/kg of highly purified β-FXIIa. Thus it can be calculated that ppNPP and highly purified β-FXIIa represent 157-fold and 18,333-fold purification of human plasma NPP.

Dose-matched cardiovascular effects The cardiovascular responses to human NPP (5.5 mg/kg), ppNPP (35 µg/kg), and β-FXIIa (300 ng/kg) were similar with respect to peak incre-

ments of SBP, DBP, and HR, such that any minor differences were not statistically significant (Table 1). The SBP increments peaked at about 45–50 mmHg, all DBP increments peaked at about 20 mmHg, and all HR increments peaked at about 70–90 beats/min.

Representative BP responses. Normal (untrypsinized) human plasma (20 µl plasma equivalent or 5.5 mg/kg) injected into bioassay rats produced no discernable change in blood pressure (arrow, Fig. 7*A*). Human NPP (5.5 mg/kg) produced a characteristic (biphasic or multiphasic) BP response, mainly a slight depressor followed by a large pressor, lasting for ~15 min (Fig. 7*B*). Human ppNPP (35 µg/kg) and coagulation β-FXIIa (300 ng/kg) both produced BP response profiles that were very comparable to that of human NPP (Fig. 7, *C* and *D*).

Effects on plasma catecholamines. The effect of human NPP, ppNPP, and coagulation β-FXIIa on the plasma catecholamines epinephrine, norepinephrine, and dopamine were compared in bioassay rats (Table 2). Human NPP (5.5 mg/kg) increased epinephrine from 169 ± 61 pg/ml at baseline to a peak of 8,292 ± 409 pg/ml ($P < 0.001$) and norepinephrine from 69 ± 23 to 480 ± 76 pg/ml ($P < 0.001$), with no change in DA. The ratio of epinephrine to norepinephrine at baseline was 2.5:1, and at the peak it increased to 17.3:1. Similarly, both ppNPP (35 µg/kg) and coagulation β-FXIIa (300 ng/kg) increased epinephrine and norepinephrine significantly ($P < 0.001$) at the peak response compared with their respective baselines. Again, the dopamine levels did not change. The ratio of epinephrine to norepinephrine at peak response for ppNPP increased from 6.4:1 to 20.7:1, and for coagulation β-FXIIa it increased from 5.1:1 to 17.9:1. Human NPP peak epinephrine was also higher than the peak epinephrine given by ppNPP and β-FXIIa ($P < 0.05$).

DISCUSSION

The pressor effect of NPP was first observed in rat and human plasmas that had undergone controlled tryptic activation, as required for the activation of prorenin (21). Such trypsin-activated plasma preparations (10–20 µl of plasma equivalent, intravenously) produced impressive pressor and
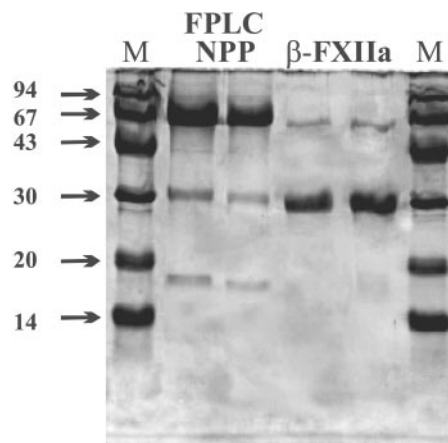

Fig. 5. SDS-PAGE electrophoresis of FPLC-NPP and commercial β-FXIIa. SDS-PAGE (17.5% acrylamide gel) of FPLC-NPP (30 µg protein load/track) and highly purified human coagulation β-FXIIa (20 µg protein load/track). Molecular markers (M, in kDa, MATERIALS AND METHODS) are shown at the arrows.
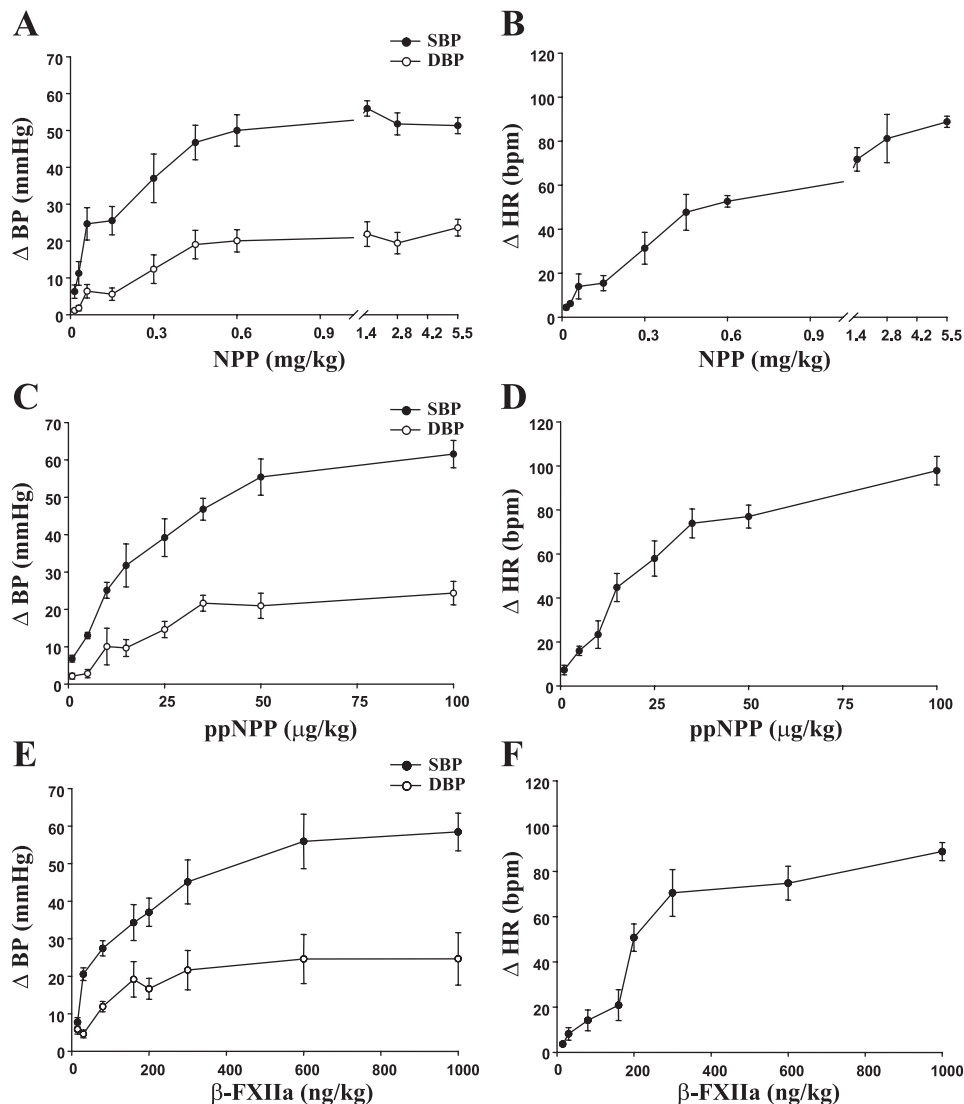
Fig. 6. Dose-response curves obtained from injections of NPP, ppNPP, and human coagulation β-FXIIa. Human NPP (mg/kg iv, $n = 10$), ppNPP (μg/kg iv, $n = 8$), and highly purified human β-FXIIa (β-FXIIa, ng/kg iv, $n = 6$). Increments of systolic and diastolic blood pressures (SBP and DBP, mmHg) and heart rate [HR, beats/min (bpm)] are shown with the use of bioassay rats. Values are means ± SE. Control injections of normal human plasma (20 μl/rat iv) not activated with trypsin or of physiological saline at 50 μl/rat does not raise BP or HR.

heart rate responses in ganglion-blocked (pentolinium) bioassay rats (18, 21). Human and rat NPPs appeared to be roughly equipotent. Administration of the angiotensin-converting enzyme inhibitor captopril (or enalapril) caused an unexpected, impressive potentiation of the effects of NPP, raising questions as to the mechanism involved (18, 19, 21, 25).

Emerging evidence suggested that the potency of NPP depended on its initiation and recruitment of mechanisms acting synergistically in the body to exert the observed effects (19, 21, 25), making it necessary to continue using whole animal bioassays rather than simpler in vitro techniques such as isolated organs, tissues, or cells. Impure NPP preparations were investigated when no others were available, and the active agent had not been identified or isolated. Rat bioassays were the only means of measurement, and they gave encouragingly reproducible results (19, 25), which contributed to a growing comprehension of this apparently novel paradigm. Studies using plasma preparations that were not completely pure would still have retained doubt as to the active agent(s) responsible for the bioactivity of NPP.

After strong sequence homology of human plasma NPP with human β-FXIIa was demonstrated (21), several questions still remained. First and foremost, the literature on β-FXIIa provided no grounds for associating it with the pressor and cardiac stimulatory properties of NPP. Second, the literature indicated that, if anything, β-FXIIa might be expected to produce depressor, rather than pressor effects, because of its linkage to the kallikrein-kinin system and its potential for generating endogenous bradykinin (1, 30, 31).

Table 1. *Blood pressures from comparable doses of NPP, ppNPP, and commercial coagulation β-FXIIa*

	ΔSBP, mmHg	ΔDBP, mmHg	ΔHR, beats/min
Human NPP	51±2	24±2	89±3
ppNPP	47±3	22±2	74±7
Purified β-FXIIa	45±6	22±5	70±10

Values are nonsignificant and expressed as means ± SE. Human new pressor protein (NPP) (5.5 mg/kg iv, $n = 10$), partially purified NPP (ppNPP, 35 μg/kg iv, $n = 8$), and highly purified β-FXIIa (β-FXIIa, 300 ng/kg iv, $n = 8$) increased systolic/diastolic blood pressures (SBP/DBP) and heart rate (HR) comparably when injected into bioassay rats.

Fig. 7. Representative blood pressure responses to a normal, unactivated (control) human plasma preparation (NHP), and to human NPP, ppNPP, and coagulation β-FXIIa. Human NPP (5.5mg/kg iv), ppNPP (35μg/kg iv), and highly purified β-FXIIa (300 ng/kg iv) produced comparable blood pressure responses in bioassay rats lasting ~15 min. A normal (control) human plasma preparation not activated with trypsin (NHP, 20 μl/rat or 5.5 mg/kg iv) produced no change in blood pressure.
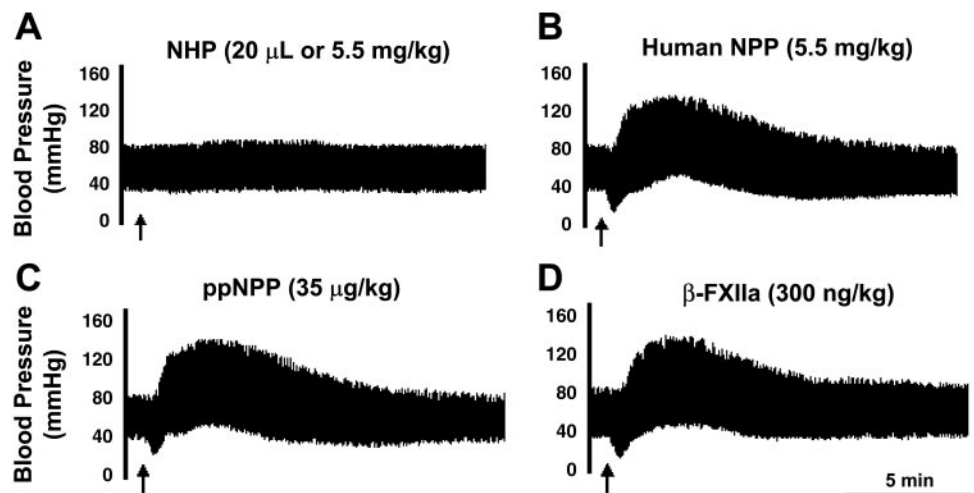
After the accumulation of evidence based on impure NPP (19, 21, 22, 25) and on an earlier preparation of commercial β-FXIIa (18, 22), a more highly purified β-FXIIa prepared from pure FXII became available (MATERIALS AND METHODS). This made it possible and timely to characterize various purities of human plasma NPP prepared in our laboratory and compare them with the commercial preparation to increase confidence that β-FXIIa was the bioactive agent in all cases.

Our data show that gel filtration (Sephadex G-100) of impure NPP removes a high proportion of unwanted protein and that NPP activity appears rather diffusely at the 30-kDa end of the elution profile (Fig. 1A). This broad elution zone of activity was pooled, desalted, lyophilized, and rerun on a Sephadex G-75 gel column, and a much sharper elution peak was obtained, most of which also represents unwanted protein (Fig. 1B). Only the 30-kDa end of this elution curve contains the NPP activity in a rather wide, shallow zone. Thus the two gel filtration procedures remove a large bulk of unwanted plasma proteins but yield no distinct protein peak representing NPP activity. These active fractions were pooled and prepared as described (MATERIALS AND METHODS) and designated ppNPP, which on electrophoresis, produced no clearly visible band corresponding to β-FXIIa at 30 kDa (Fig. 2), indicating that the purity was still low.

Accordingly, the ppNPP preparation was further purified by using a FPLC chromatography system (MATERIALS AND METHODS), and most of the NPP activity eluted as a fairly distinct protein peak (with shoulders) centered on fraction 37 (Fig. 3). The NPP activity occurred in fewer fractions (33–42; Fig. 3) compared with gel filtration (fractions 70–100, Fig. 1B). When the commercial β-FXIIa was similarly eluted by using FPLC, its activity peak centered on fraction 38 (Fig. 4). Thus the ppNPP activity (fractions 33–42) and commercial β-FXIIa (fractions 32–42) eluted almost identically, suggesting that proteins having very similar anionic charge and activity were involved in both cases, most likely β-FXIIa.

When this additionally purified FPLC-NPP preparation was electrophoresed on a SDS-PAGE gel, three distinct bands were observed, one of them corresponding to 30 kDa, indicating that substantial purification beyond the gel filtration stage had been achieved (Fig. 5 vs. Fig. 2, ppNPP). Here a high load of β-FXIIa was applied (20 μg protein/track), and it yielded a very distinct band corresponding to 30 kDa, with a minor band appearing at a higher molecular mass between 43 and 67 kDa (Fig. 5). This higher molecular mass contaminant is probably α-FXIIa because: 1) it is an expected activation product of FXII (18), 2) its molecular mass approximates that of α-FXIIa (50 kDa), and 3) the supplier specified it as the minor contaminant of β-FXIIa (MATERIALS AND METHODS).

The fact that the higher molecular mass contaminant was evident when a high load of β-FXIIa was applied (20 μg/track, Fig. 5), but not when the lower but considerable loads of 5 or 10 μg/track were applied (Fig. 2), suggests that the contaminant was present at a very low concentration. It only became visible when loaded in high concentrations, and this supports the supplier's claim of high purity (>90%, MATERIALS AND METHODS).

Table 2. *Effects of human NPP, ppNPP, and commercial β-FXIIa on plasma catecholamine levels*

	E, pg/ml	NE, pg/ml	DA, pg/ml	E:NE
Human NPP (20 μl or 5.5 mg/kg iv)				
Baseline	169±61	69±23	33±4	2.5:1
Peak	8,292±409*†	480±76*	55±7	17.3:1
Δ Peak	8,123±398*†	412±60*	22±7	19.7:1
Recovery	933±380*†	108±39	37±9	8.6:1
ppNPP (35 μg/kg iv)				
Baseline	148±24	23±4	29±7	6.4:1
Peak	7,025±466*	340±51*	46±4	20.7:1
Δ Peak	6,877±433*	317±47*	18±5	21.7:1
Recovery	201±37	27±3	24±4	7.4:1
Coagulation β-FXIIa (300 ng/kg iv)				
Baseline	147±30	29±4	31±10	5.1:1
Peak	6,443±496*	360±73*	55±7	17.9:1
Δ Peak	6,296±456*	330±70*	25±7	19.1:1
Recovery	175±42	48±15	38±7	3.6:1

Values are means ± SE. Plasma epinephrine (E), norepinephrine (NE), and dopamine (DA) levels (pg/ml) before and after injection of human NPP (5.5 mg/kg, $n = 8$), ppNPP (35 μg/kg, $n = 8$), and highly purified coagulation β-FXIIa (300 ng/kg, $n = 8$) into bioassay rats. Values are from blood samples taken at baseline (before injection), at peak SBP response, and after return of SBP to baseline. *$P < 0.001$ vs. corresponding baseline levels within the group; †$P < 0.05$ NPP vs. corresponding values given by ppNPP and β-FXIIa.

The 30-kDa bands in the gel tracks of FPLC-NPP and of commercial β-FXIIa (Fig. 5) yielded amino acid sequences specific to the heavy chain of the β-fragment of human FXII, as determined by mass spectrometry (RESULTS). Such positive verification of β-FXIIa in the relevant 30-kDa bands taken from both commercial β-FXIIa and FPLC-NPP strongly suggests that the bioactivity in both preparations is attributable to β-FXIIa. The band at about 67 kDa in the FPLC-NPP gel track yielded sequences that were characteristic of human serum albumin (RESULTS).

Dose-response curves constructed using impure NPP, ppNPP, and commercial β-FXIIa all exhibited very similar pressor response profiles highly characteristic of NPP as described (RESULTS, Figs. 6 and 7). It is unclear why the HR continued to increase noticeably into the higher dose range after the concurrent SBP and DBP responses had leveled off (Fig. 6).

Similar SBP, DBP, and HR response profiles across the dose-response curves support the probability that a single active agent was involved throughout, i.e., β-FXIIa. Any other bioactive contaminants would almost certainly have been present in different quantities and proportions in each preparation, causing the response profiles to vary at different doses, especially at the lowest doses where the concentration of a minor impurity would drop to a trivial level. In the case of NPP, the starting material was plasma and any contaminants in such plasma-derived NPP preparations, which must have varied considerably under directional purification protocols, were aimed at concentrating β-FXIIa and not any contaminants. Thus similar effects produced by such dissimilar preparations, at low and high doses, (Fig. 6) argue in favor of β-FXIIa being the prime, if not only, active agent throughout. A multiplicity of bioactive contaminants, differing in kind and quantity from preparation to preparation, relative to their content of β-FXIIa, would not likely have produced such similar response profiles across the full range of doses.

On the other hand, highly purified β-FXIIa was derived from highly purified human FXII and should be substantially devoid of other plasma protein impurities. As stated above, the supplier specified a minor contaminant to be α-FXIIa (MATERIALS AND METHODS), which is supported by our electrophoretic evidence showing no discernible contaminant when β-FXIIa is loaded at 5 or 10 μg/track (Fig. 2) and only a minor contaminant when loaded at 20 μg/track (Fig. 5). This minor contaminant is located between the molecular mass markers of 43 and 67 kDa, which could agree with the molecular mass of α-FXIIa (50 kDa). The latter molecule does not incorporate the β-FXIIa (30 kDa) moiety encompassing the serine protease catalytic site (4, 11, 28), which is most probably responsible for the bioactivities we attribute to β-FXIIa. Thus if the main, or sole, minor contaminant of the β-FXIIa preparation is α-FXIIa, which lacks the catalytic site responsible for the observed bioactivity β-FXIIa, then α-FXIIa is not likely to be responsible.

The doses of NPP, ppNPP, and β-FXIIa used for further study were chosen in terms of equivalence of ΔSBP, ΔDBP, and ΔHR responses in bioassay rats. The impure NPP was selected for continuity with past experiments, i.e., 20 μl plasma equivalent per ~300-g rat, which translates into 5.5 mg protein/kg and is comparable to ppNPP at 35 μg/kg and to commercial β-FXIIa at 300 ng/kg (Figs. 6 and Table 1).

These doses elicited individual blood pressure responses that closely resembled each other in all details, i.e., characteristic biphasic/multiphasic profiles lasting about 15 min, a short, small depressor phase at the outset, and similar peak SBP, DBP, and HR increments (Fig. 7). Such similarities are supported statistically (Table 1) and provide more evidence in favor of β-FXIIa. It is noteworthy that untrypsinized normal human plasma elicits no NPP response (Fig. 7B), supporting the view that NPP is not necessarily present in the circulation until FXII is activated.

An important question is whether enough coagulation FXII is present in normal human plasma to deliver sufficient β-FXIIa on activation to elicit cardiovascular effects similar to those observed here. The normal concentration of FXII is reported to be about 30 μg/ml (9, 11). On this basis, 20 μl plasma equivalent (or 5.5 mg of protein/kg) could provide a total of 600 ng of the precursor FXII. Assuming stoichiometric conversion of FXII to β-FXIIa and allowing for the lower molecular mass of β-FXIIa, even this minute volume should be able to provide sufficient β-FXIIa to elicit the blood pressure and HR responses seen in Fig. 6, and the same applies to the corresponding catecholamine responses in Table 2. Obviously, higher volumes of activated plasma could deliver larger quantities of β-FXIIa in vitro, and the same could be expected in vivo under conducive conditions (see below).

Plasma catecholamine determinations, at the peak SBP response to all three preparations (NPP, ppNPP, and β-FXIIa) produced massive increases in epinephrine levels (up to 50-fold) but more modest increases in norepineprine (about 10-fold), thereby increasing epinephrine-to-norepinephrine ratios from 5:1 to 20:1 (Table 2). The dopamine values were relatively low and did not change much throughout. The impure NPP elicited a significantly higher (8,292 ± 409 pg/ml) plasma epinephrine concentration than ppNPP (7,025 ± 466 pg/ml) and β-FXIIa (6,433 ± 496 pg/ml). These gross increments were only modestly higher than the net increments (Table 2).

Bearing in mind that highly accurate dose equivalence is difficult to calculate or achieve when using diverse preparations that produce complex effects, the higher epinephrine concentration given by impure NPP could be explained in quantitative, rather than qualitative, terms. Doses selected in terms of ability to raise SBP/DBP and HR (Figs. 6 and Table 1) need not necessarily trigger adrenal catecholamine release with exactly the same effectiveness (Table 2). Therefore, the observed greater effect of NPP on catecholamines relative to the other preparations need not contradict the view that β-FXIIa was the bioactive agent operative throughout. NPP may simply have contained more β-FXIIa than was intended for an exact match with the other preparations.

We have recently reported evidence linking the presence of plasma NPP activity to elevated blood pressure in anephric patients undergoing hemodialysis (22). Such evidence supports the probability that NPP activity is not only formed in the body but may contribute effects similar to those produced by administered NPP and coagulation β-FXIIa. The coagulation FXII cascade is readily activated under physiological conditions involving inflammation or injury (6). It can certainly occur under pathophysiological conditions involving essential hypertension (16), coronary heart disease (5, 32), severe sepsis and septic shock (7, 10), myocardial infarction and coronary atherosclerosis (13), systemic inflammation (2), end-stage chronic

renal failure (17), hemodialysis (8, 27), hemolytic uremic syndrome (20), diabetes (3), and ischemia-reperfusion injury (26). Thus endogenous production of coagulation β-FXIIa is to be expected, in which case it could become available to exert effects similar to those using exogenous β-FXIIa. As stated at the beginning of this study, these effects are potentiated by treatment with angiotensin-converting enzyme inhibitors and could interfere with their beneficial therapeutic effects.

In conclusion, our data strongly support the hypothesis that our three preparations representing various purities of human plasma NPP elevate SBP, DBP, HR, and plasma catecholamines primarily, if not exclusively, through their content of coagulation β-FXIIa. Such cardiovascular and sympathoadrenal actions of β-FXIIa propose a novel axis linking the coagulation FXII cascade to cardiovascular regulatory mechanisms.

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