Angiotensin converting enzyme-regulated, noncholinergic sympathoadrenal catecholamine release mediates the cardiovascular actions of human 'new pressor protein' related to coagulation beta-factor XIIa

Peter C Papageorgiou PhD^{1,2,3}, Demetrios Simos MSc MD¹, Frans Boomsma PhD⁴,
Rasmus Rojkaer PhD⁵, Daniel H Osmond PhD^{1,2}

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BACKGROUND: Human 'new pressor protein' (NPP), related to coagulation beta-factor XIIa (β -FXIIa), potentially releases sympathoadrenal catecholamines in bioassay rats, with concurrent elevation of systolic and diastolic blood pressure (SBP/DBP) and heart rate (HR). Elevated plasma NPP/ β -FXIIa levels in hypertensive anephric pediatric patients on hemodialysis associated with fluid status and blood pressure changes were previously reported, suggesting that NPP/ β -FXIIa contributed to their hypertension.

OBJECTIVE: To investigate the mechanism of action of NPP/ β -FXIIa.

METHODS: Hemodynamic and sympathoadrenal responses to NPP (20 μ L plasma equivalent/rat) or coagulation β -FXIIa (300 ng/kg intravenously) were measured in rats treated with pentolinium (ganglion blockade [+GB]) and/or captopril (+CAP; angiotensin converting enzyme [ACE] inhibition).

RESULTS: In controls not receiving GB or CAP (–GB–CAP), NPP/ β -FXIIa raised plasma epinephrine (E) sixfold, SBP/DBP by 14/8 mmHg and HR by 15 beats/min. With blockade of the cholinergic pathway to the sympathoadrenal system (+GB), basal E, norepinephrine (NE), SBP, DBP and HR all dropped. However NPP/ β -FXIIa remained capable of raising E 20-fold, NE fourfold, SBP/DBP by 27/11 mmHg and HR by 20 beats/min, suggesting that it acted through a 'noncholinergic' mechanism. With +CAP alone, NPP/ β -FXIIa raised plasma E 18-fold, NE threefold, SBP/DBP by 29/8 mmHg and HR by 73 beats/min, implicating an ACE-regulated 'peptidergic' mechanism. Combining +GB with +CAP potentiated NPP/ β -FXIIa actions further by raising E 50-fold, NE sevenfold, SBP/DBP by 55/20 mmHg and HR by 87 beats/min, strengthening the efficacy of this alternate pathway.

CONCLUSIONS: The cardiovascular effects of NPP/ β -FXIIa are considerably mediated by a noncholinergic (peptidergic) ACE-regulated mechanism for sympathoadrenal catecholamine release that is enhanced by +GB and/or +CAP. Under inflammatory procoagulant conditions, endogenously produced NPP/ β -FXIIa may interfere with the antihypertensive effects of ACE inhibition therapy.

Key Words: ACE inhibitors; Bradykinin; Coagulation β -FXIIa; Factor XII; Hypertension; Sympathoadrenal

La libération de catécholamine sympathoadrénérique non cholinergique régulée par l'enzyme de conversion de l'angiotensine assure la médiation des actions cardiovasculaires de la « nouvelle protéine pressive » humaine liée au facteur bêta XIIa de la coagulation

HISTORIQUE : La « nouvelle protéine pressive » (NPP) humaine liée au facteur bêta XIIa de la coagulation (β -FXIIa) libère énormément de catécholamines sympathoadrénériques dans des essais biologiques sur des rats, conjointement à une élévation de la tension artérielle systolique et diastolique (TAS et TAD) et de la fréquence cardiaque (FC). On a déjà déclaré des taux plasmatiques élevés de la NPP et du β -FXIIa chez des patients pédiatriques anéphriques et hypertensifs sur hémodialyse, associés à des modifications de l'état liquidien et de la tension artérielle, laissant supposer que la NPP et le β -FXIIa contribuaient à leur hypertension.

OBJECTIF : Explorer le mécanisme d'action de la NPP et du β -FXIIa.

MÉTHODOLOGIE : Les auteurs ont mesuré les réponses hémodynamique et sympathoadrénérique à la NPP (20 μ L d'équivalent plasma/rat) ou au β -FXIIa à la coagulation (300 ng/kg par voie intraveineuse) chez des rats traités au pentolinium (gangliobloqueur [GB]) ou au captopril (CAP; inhibition de l'enzyme de conversion de l'angiotensine [ECA]).

RÉSULTATS : Chez les sujets témoins qui ne prenaient ni GB ni CAP, la NPP et le β -FXIIa ont sextuplé l'adrénaline plasmatique et ont augmenté la TAS et la TAD de 14 mmHg et de 8 mmHg, respectivement, ainsi que la FC de 15 battements/min. Lorsque les voies cholinergiques vers le système sympathoadrénérique étaient bloquées (GB), tant l'adrénaline basale, la noradrénaline, la TAS, la TAD que la FC diminuaient. Cependant, la NPP et le β -FXIIa pouvaient encore multiplier l'adrénaline par vingt, quadrupler la noradrénaline, accroître la TAS et la TAD de 27 mmHg et de 11 mmHg, respectivement, et la FC de 20 battements/min, laissant supposer qu'elles fonctionnaient selon un mécanisme non cholinergique. Avec l'ajout de CAP seul, la NPP et le β -FXIIa multipliaient l'adrénaline par 18, triplait la noradrénaline, accroissait la TAS et la TAD de 29 mmHg et de 8 mmHg, respectivement, et la FC de 73 battements/min, mettant en cause un mécanisme peptidergique régulé par l'ECA. L'association de GB et de CAP stimulait l'action de la NPP et du β -FXIIa encore davantage, multipliant l'adrénaline par 50, la noradrénaline par sept, accroissant la TAS et la TAD de 55 mmHg et de 20 mmHg, respectivement, et la FC de 87 battements/min, ce qui renforce l'efficacité de cette autre voie.

CONCLUSIONS : Les effets cardiovasculaires de la NPP et du β -FXIIa sont considérablement médiés par un mécanisme non cholinergique (peptidergique) régulé par l'ECA qui libère des catécholamines sympathoadrénériques stimulées par les GB, le CAP ou ces deux médicaments. Dans des conditions procoagulantes inflammatoires, la NPP et le β -FXIIa à production endogène peuvent nuire aux effets antihypertenseurs de la thérapie aux inhibiteurs de l'ECA.

¹Departments of Physiology and Medicine; ²Heart and Stroke/Richard Lewar Centre of Excellence; ³Institute of Medical Science, University of Toronto, Toronto, Ontario; ⁴Department of Internal Medicine, Erasmus Medical Centre, Rotterdam, The Netherlands; ⁵Novo Nordisk Research US, North Brunswick, New Jersey, USA

Correspondence: Dr Peter C Papageorgiou, University of Toronto, Heart and Stroke/Richard Lewar Centre of Excellence, 150 College Street, Room 67, Toronto, Ontario M5S 3E2. Telephone 416-946-8111, fax 416-946-8380, e-mail peter.papageorgiou@utoronto.ca

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Human 'new pressor protein' (NPP) is an extrarenal, approximately 30 kDa anionic enzyme that potently elevates blood pressure (BP), heart rate (HR) and sympathoadrenal catecholamines in bioassay rats acutely (1). Plasma catecholamine levels rise concurrently, predominantly of adrenal medullary origin, with epinephrine (E) levels rising more than norepinephrine (NE) levels (1-3). The cardiovascular effects of NPP depend substantially on catecholamines, as observed following adrenergic blockade with phentolamine and propranolol (2), presumably via a peptide-mediated sympathoadrenal pathway (3,4). Pretreatment of bioassay animals with an angiotensin converting enzyme (ACE) inhibitor (eg, captopril [CAP]) augment these effects (1-6), implicating ACE-dependent peptide-mediators of catecholamine release, whose bioactivity is enhanced by CAP (7). We have shown that such NPP peptide mediators include pituitary adenylate cyclase activating polypeptide (PACAP) (3) and bradykinin (BK) (4). Unexpectedly, ganglionic cholinergic blockade with pentolinium (GB) does not block the cardiovascular actions of NPP but significantly enhances them, suggesting an unexplained potentiation of its mechanism of action. Biochemically, NPP exhibits strong sequence homology with the beta-fragment of human coagulation factor XIIa (β -FXIIa), an activation product of coagulation FXII, as confirmed by mass spectrometry (1,6). This relationship is supported by evidence showing that purified β -FXIIa duplicates the properties of NPP (5,6), justifying its designation as coagulation β -FXIIa.

It is well recognized that inflammatory and procoagulant conditions trigger the endogenous activation of FXII to its derivative products (including β -FXIIa) and that clinical hemodialysis represents such a condition (8-14). We have documented high plasma FXIIa levels and NPP activity in anephric pediatric hemodialysis patients who had unexplained hypertension (15). These levels varied in relation to changes in BP and fluid status in a pattern that suggested a causal relationship (15). Such preliminary clinical observations are in accordance with extensive experimental evidence that NPP/ β -FXIIa functions as a product of activated FXII that somehow triggers sympathoadrenal activation and stimulates cardiac function to raise BP (1-6,15).

Our earlier studies have documented the modifying effects of GB and/or CAP on NPP/ β -FXIIa actions in a descriptive way, with a prime focus on the complex biochemical and physiological properties of NPP itself. The objectives of the present study were to exploit the effects of these modifiers quantitatively to elucidate the mechanism of action of NPP/ β -FXIIa, and to further establish their functional co-identity in terms of their cardiovascular and sympathoadrenal properties.

METHODS

Human NPP preparation

Human plasma considered to be normal, but unsuitable for transfusion, was provided by Canadian Blood Services in Toronto (Ontario) and activated as previously reported (1,5,6). NPP was administered intravenously (IV) to bioassay rats as a bolus dose expressed as 20 μ L of human plasma equivalent per rat. The potency of this preparation relative to more purified preparations has been well documented (5,6). It has also been demonstrated that the effects of the present NPP preparation are primarily, if not exclusively, attributable to its content of coagulation β -FXIIa (6).

Human coagulation β -FXIIa preparation

Highly purified coagulation β -FXIIa (30 kDa, Lot β -FXIIa 1000 P, Enzyme Research Laboratories, USA) was obtained at a concentration of 1.03 mg/mL dissolved in a 4 mM sodium acetate-hydrochloride and 0.15 M sodium chloride buffer (pH 5.3), and diluted with physiological saline (0.9% sodium chloride) to a final concentration of 10 ng/ μ L. The purity of this commercial preparation was certified as greater than 90% alpha-FXIIa (30 kDa) with less than 10% β -FXIIa (50 kDa) (6). Coagulation β -FXIIa was injected by IV as a bolus into bioassay rats at 300 ng/kg.

Surgical procedures

All animals were cared for and used in accordance with the principles and guidelines outlined by the Canadian Council on Animal Care. Male Wistar rats, weighing approximately 250 g to 350 g (Charles River Laboratories, Canada) were anesthetized with a 100 mg/kg intraperitoneal injection of Inactin (Promonta, Germany). Only rats that sustained minimal collateral tissue trauma and blood loss during surgery and had stable BP and HR were used for experiments.

Rat bioassay model – direct arterial BP and HR recordings: Bioassay rats were prepared as previously described (1-6), with direct arterial SBP/DBP and HR continuously recorded from a common carotid arterial cannula using a Statham DC pressure transducer (Hato Rey, USA). IV injections were made via an indwelling femoral vein cannula, without ligation to allow free venous return and were flushed with 0.1 mL of physiological saline.

Acute bilateral adrenal medullectomy: Bilateral flank incisions were made above each kidney and each adrenal gland was exposed, the cortex cut open and the medullary core gently squeezed out, leaving the cortical layer in place (2). The muscle and skin layers were sutured with 3-0 silk ligature (Ethicon Inc, USA), and the rats were reconnected to the recording system and allowed to stabilize for 10 min to 15 min.

Spinal transection between C6 and C7: Rats anesthetized with Inactin were positioned in a stereotaxic frame (David Kopf Instruments, USA) to immobilize their cranium and hold their spinal column taut (16-19). An incision was made in the dorsal neck region and the cervical bones (C5 to C7) were exposed by blunt dissection. Under magnification (25 $\times$, Olympus B201, Germany), bone crushers were used to remove the vertebrae and expose the spinal column between C6 and C7. The exposed cord was completely ablated by gentle suction using a laboratory-made vacuum apparatus. A cautery unit (model #150, Geiger Medical Technologies Inc, USA) was used to control bleeding. The muscle and skin layers were closed using 3-0 silk sutures and the animals were allowed to recover on a mechanical ventilator delivering room air at 2 cc per 100 g body weight. The rats were then reconnected to the recording system and allowed to stabilize for 10 min to 15 min.

Plasma catecholamine and BK metabolite 1-5 determinations

Arterial blood samples (1 mL each) were withdrawn from the carotid artery cannula and collected into heparinized saline (100 μ L) containing glutathione (2.4 mg/mL, Boehringer Mannheim, Germany), to prevent coagulation and oxidation of catecholamines, as previously described (2,6). The samples were separated in a refrigerated centrifuge, divided into aliquots and stored at -40°C until the time of catecholamine and BK 1-5 assay.

The first (baseline) blood sample was taken 10 min before the injections of NPP or β -FXIIa and the second (peak) sample was drawn at the peak of the systolic BP (SBP) pressor response (approximately 2 min). Approximately 15 min before withdrawal of each sample from the bioassay rat, 1 mL of blood from a donor rat was infused to avoid hypovolemia at the time of blood collection (2,3,6). The donor rats (approximately 350 g) were prepared in the same way as the bioassay rats and their fluid volume was maintained by injecting 1 mL of physiological saline 10 min to 15 min before the removal of each blood sample. Each bioassay rat contributed two 1 mL blood samples while donor rats contributed four 1 mL samples, with recovery periods between samplings.

Plasma levels of E and NE were determined using high-performance liquid chromatography with fluorimetric detection (20). This method was sensitive enough to analyze plasma volumes of approximately 250 μ L and detect pg/mL concentrations of plasma catecholamines.

Plasma levels of the stable BK metabolite BK 1-5 (Arg-Pro-Gly-Phe) were determined in duplicate by an enzyme immunoassay kit (Markit-M BK 1-5, Dainippon Pharmaceuticals Ltd, Japan) as described by the manufacturer. The enzyme immunoassay kit used an anti-BK 1-5 antibody (100% reactivity) that had negligible cross-reactivity with intact BK (BK 1-9, 0.02%) or with its other metabolites (BK 1-8, 0.01%; BK 1-7, 0.01%; BK 1-6, 0.60%) (21-23).

Figure 1 Effects of human new pressor protein (NPP) on systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR). NPP was injected at 0 min, and SBP, DBP and HR were recorded continuously and presented in 0.5 min intervals. Data presented as means and SEMs. Group 1 received no ganglion blockade with pentolinium (GB) or captopril (CAP) (-GB-CAP, n=15). Group 2 received no GB but received CAP (-GB+CAP, n=16). Group 3 received GB but did not receive CAP (+GB-CAP, n=14). Group 4 received both GB and CAP (+GB+CAP, n=15). * $P < 0.05$ for SBP, DBP and HR versus the corresponding baseline value

Treatment agents

The cholinergic GB agent pentolinium tartrate (P-3520, Sigma-Aldrich, USA) was prepared in polyvinylpyrrolidone (PVP-40T, Sigma-Aldrich) and injected subcutaneously to allow for its gradual release during the experimental period, as described previously (1-6). A stock solution (10 mg/mL) of the ACE inhibitor CAP (C-4042, Sigma-Aldrich) was prepared fresh in physiological saline every experimental day and injected by IV as indicated below. All agents were kept on ice throughout the experimental period.

Experimental protocol and treatment groups

The experimental animals were divided into four treatment groups: group 1 rats received no GB (pentolinium) or CAP (control, -GB-CAP); group 2 rats received no GB but received CAP (-GB+CAP); group 3 rats received GB but did not receive CAP (+GB-CAP); and group 4 received both GB and CAP (+GB+CAP).

GB was induced using pentolinium (19.2 mg/kg subcutaneously) and CAP (2.5 mg/kg IV) (1,5). The peptides angiotensin (Ang) I (A-9650, Sigma-Aldrich) and Ang II (A-9525, Sigma-Aldrich) were injected (60 ng/kg IV) before and at least 40 min after CAP was given to test for obliteration of the pressor response to Ang I without reduction of the pressor responsiveness to Ang II, thereby verifying the effectiveness of ACE inhibition induced by CAP.

The effects of acute bilateral adrenal medullectomy (2MDX) and spinal transection (SPT) were also investigated (ie, 2MDX in the

+CAP groups [-GB+CAP+2MDX and +GB+CAP+2MDX] and SPT in groups 3 and 4 [-GB-CAP+SPT, +GB-CAP+SPT, +GB+CAP+SPT]). Note that in group 3, cholinergic blockade was induced by +GB, +SPT or both.

In all experiments, human NPP was injected as a bolus dose of 20 μ L plasma equivalent per rat (IV) and human coagulation β -FXIIa as a bolus dose of 300 ng/kg (IV) (6). Any repeat injections were made only after the BP and HR responses to the previous injection had returned to baseline, usually after 15 min to 20 min.

Data handling and statistical analyses

All results are expressed as mean $\pm$ SEM of measured baseline values and peak increments obtained after injections of human NPP or β -FXIIa. Each datum point represents the average of multiple determinations, as indicated. Statistical comparisons of changes in SBP, diastolic BP (DBP) and HR (Figure 1; time 0.5 min to 15 min) from baseline values (at time 0 min) were analyzed using one-way repeated measures ANOVA with Dunnett's correction for multiple comparisons using the Sigma Stat program (v 2.03, Systat Software Inc, USA) and the probability values indicated. All delta (Δ) values (Δ SBP, Δ DBP, Δ HR and Δ catecholamines) were calculated by subtracting peak values from their corresponding baseline values. Time intervals (Figures 2 and 3; time 0 min to 15 min) were statistically compared (every 0.5 min) with the corresponding value of the other relevant treatment group using an unpaired two-tailed distribution Student's *t* test. All

Figure 2 The effect of captopril (CAP) on the action of human new pressor protein (NPP) on the change in systolic blood pressure (Δ SBP), the change in diastolic blood pressure (Δ DBP) and the change in heart rate (Δ HR). Human NPP was injected at 0 min and compared among treatment groups. All data represent an average of Δ SBP, Δ DBP and Δ HR every 30 s, and are presented as means and SEMs. * $P < 0.05$ for CAP treatment (+CAP) versus the corresponding no CAP treatment (-CAP) values. -GB No ganglion blockade with pentolinium; +GB Ganglion blockade treatment; vs Versus

other comparisons within and between treatment groups were analyzed using one-way repeated measures ANOVA with Duncan's correction for multiple comparisons. $P \leq 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of CAP, GB, 2MDX and SPT treatments on basal BP and HR
The effect of CAP, GB, 2MDX and SPT treatments on basal BP and HR are presented in Table 1. In group 1 (control, -GB-CAP), basal pressures (SBP/DBP) of bioassay rats stabilized at 129/90 mmHg and their HR at 377 beats/min. CAP alone (group 2, -GB+CAP) dropped the basal pressures and increased basal HR while GB alone (group 3, +GB-CAP) further lowered both basal pressures and HR. In combination (group 4, +GB+CAP), there was no additional drop in SBP but DBP fell slightly while HR increased. 2MDX did not lower basal BP and HR significantly in the -GB+CAP+2MDX rats (group 2) relative to the corresponding control group but did so in the +GB+CAP+2MDX rats (group 4). SPT alone (group 3, -GB-CAP+SPT) had the same effect as GB alone and did not magnify the effect when combined with GB (group 3, +GB-CAP+SPT). Much the same is true in the presence of CAP (group 4, +GB+CAP+SPT), except that the HR was not as low as in the corresponding -CAP groups (group 3).

Figure 3 The effect of ganglion blockade (GB) on the action of human new pressor protein (NPP) on the change in systolic blood pressure (Δ SBP), the change in diastolic blood pressure (Δ DBP) and the change in heart rate (Δ HR). Human NPP was injected at 0 min and compared among treatment groups. All data represent an average of Δ SBP, Δ DBP and Δ HR every 30 s, and are presented as means and SEMs. * $P < 0.05$ for the GB treatment (+GB) versus the corresponding no GB (pentolinium) treatment (-GB) values. -CAP No captopril treatment; +CAP Captopril treatment; Vs Versus

Cardiovascular effects of human NPP in bioassay rats (Figure 1)

Group 1, -GB-CAP (n=15): In control animals, there was an initial drop in SBP/DBP within 30 s in response to NPP injection, followed by a slight increase in SBP/DBP, before the return to baseline. HR changed minimally from baseline throughout.

Group 2, -GB+CAP (n=16): With ACE inhibition using CAP, NPP injection resulted in a quick SBP rise of 29 mmHg above baseline within 1 min, in contrast to the drop in DBP by 24 mmHg within 30 s. NPP raised the HR to a peak of 77 beats/min above baseline within 3 min and it remained significantly elevated for approximately 12.5 min.

Group 3, +GB-CAP (n=14): With the cholinergic pathway blocked, there was virtually no SBP/DBP depressor effect after NPP injection. However, there was a marked SBP rise of 26 mmHg above baseline within 2 min of NPP injection and a return to baseline after 9 min. The peak HR increased by a modest 20 beats/min above the baseline.

Group 4, +GB+CAP (n=15): With both cholinergic GB and ACE inhibition, NPP induced no initial drop in SBP but a quick rise of 50 mmHg within 2 min to 3 min and remained elevated for up to 11.5 min. The DBP fell approximately 10 mmHg within 30 s, then gradually climbed to a modest peak of approximately 20 mmHg and remained above baseline for approximately 6.5 min. The peak HR increased impressively by 87 beats/min within 1 min to 2 min and remained elevated for another 9 min.

TABLE 1
Effect of the treatment conditions on basal blood pressure (BP) and heart rate (HR) in bioassay rats

	-GB-CAP (n=15)		
SBP, mmHg	129±3		
DBP, mmHg	90±4		
HR, beats/min	377±11		
	-GB+CAP (n=16)	-GB+CAP+2MDX (n=6)	
SBP, mmHg	111±4*	93±6*	
DBP, mmHg	58±2*	53±6*	
HR, beats/min	405±8*	398±18	
	+GB-CAP (n=14)	-GB-CAP+SPT (n=6)	+GB-CAP+SPT (n=6)
SBP, mmHg	81±2*†	82±4*†	83±4*†
DBP, mmHg	45±1*†	44±3*†	46±6*†
HR, beats/min	314±7*†	325±12*†	315±9*†
	+GB+CAP (n=15)	+GB+CAP+2MDX (n=8)	+GB+CAP+SPT (n=8)
SBP, mmHg	79±3*†	61±3*†‡§	73±5*†
DBP, mmHg	39±2*†‡	34±5*†‡	35±3*†‡
HR, beats/min	350±9*†‡	341±11*†‡	358±13*†‡

The basal systolic BP (SBP), diastolic BP (DBP) and HR were recorded to determine the effect of treatment agents and/or surgical procedures (captopril [CAP], ganglion blockade with pentolinium [GB], bilateral adrenal medullectomy [2MDX] and spinal transection [SPT]) in the bioassay rat. Data are presented as means ± SEMs. **P*<0.01 versus group 1; †*P*<0.01 versus group 2; ‡*P*<0.01 versus group 3; §*P*<0.05 versus corresponding variable in similar group. + Treatment; – No treatment

Identifying the effects of ACE inhibition by CAP on NPP responses (Figure 2)

Effects of +CAP in -GB animals (group 1 versus group 2): +CAP alone eliminated the initial SBP depressor phase after NPP compared with the -CAP animals and exaggerated the subsequent pressor effect for approximately 4 min, after which, it actually depressed the SBP. +CAP did not attenuate the initial DBP depressor effect of NPP but actually accentuated it after 3 min. +CAP increased the HR significantly within 2 min, which lasted for 9 min.

Effects of +CAP in +GB animals (group 3 versus group 4): +CAP magnified and prolonged NPP's effects during the first one-half of the SBP/DBP response. In addition, +CAP caused the HR to increase within 1 min and remain elevated for up to 13.5 min.

Overall +CAP effect: Overall, +CAP enhanced the cardiac effects of NPP much more prominently than its effects on SBP/DBP. +CAP generally potentiated SBP responses more than the DBP responses, especially in animals receiving GB.

Identifying the effects of cholinergic GB on NPP responses (Figure 3)

Effects of +GB in -CAP animals (group 1 versus group 3): +GB alone blunted the initial SBP/DBP depressor phase after NPP and modestly enhanced the SBP pressor responses, without much change in the HR.

Effects of +GB in +CAP animals (group 2 versus group 4): The initial depressor effect of NPP was blunted but its subsequent pressor effects on SBP/DBP were enhanced for a considerable portion of the response. NPP produced marked increments of HR throughout, however such effects were not significantly enhanced by +GB.

Overall +GB effect: Overall, +GB attenuated any initial SBP/DBP depressor effects of NPP compared with the -GB animals. +GB markedly potentiated the SBP more than the DBP pressor responses to

Figure 4) Effect of treatment conditions on the maximum change in systolic blood pressure (Δ SBP), the change in diastolic blood pressure (Δ DBP) and the change in heart rate (Δ HR) of human coagulation beta factor XIIa (β -FXIIa) and new pressor protein (NPP). Human coagulation β -FXIIa (open bars) and NPP (all other bars) were injected and the maximum response was compared among treatment conditions. Data are presented as means and SEMs. **P*<0.05 versus corresponding group 1; †*P*<0.05 versus corresponding group 2; ‡*P*<0.05 versus corresponding group 3; §*P*<0.05 versus corresponding -2MDX group (group 2 or 4). + Treatment; – No treatment; 2MDX Bilateral adrenal medullectomy; CAP Captopril; GB Ganglion blockade with pentolinium; SPT Spinal transection

NPP in all situations except for the DBP of the -CAP animals (upper panel). HR was unaffected in the -CAP animals by NPP (upper panel), as opposed to the considerable HR increase in the +CAP animals (lower panel); however, +GB had no modifying effect in either.

Effects of 2MDX and cholinergic GB by SPT on maximum Δ BP and Δ HR responses to NPP (Figure 4)

2MDX significantly attenuated the Δ BP and Δ HR responses to NPP (groups 2 and 4). The effectiveness of cholinergic GB by C6 to C7 surgical SPT was compared with pharmacological blockade with pentolinium (+GB) (groups 3 and 4). The effects of +GB or +SPT alone, or combined (+GB+SPT) were comparable on Δ BP and Δ HR responses to NPP (group 3). Combining +GB+CAP or +GB+CAP+SPT similarly potentiated the effects on Δ BP and Δ HR (group 4).

Comparison of maximum Δ BP and Δ HR responses of human NPP and coagulation β -FXIIa (Figure 4)

The maximum effects of highly purified human coagulation β -FXIIa (open bars) on Δ BP and Δ HR were compared with those of human

Figure 5 Bradykinin metabolite levels of human coagulation beta factor XIIa (β -FXIIa) and human new pressor protein (NPP). Bradykinin 1-5 after injections of human coagulation beta factor XIIa (β -FXIIa; $n=6$) or human new pressor protein (NPP; $n=6$) at baseline (Pre) and at peak response (Post) in group 3 (received ganglion blockade [pentolinium] but no captopril) bioassay rats. Data presented as means and SEMs. * $P<0.01$ peak response versus baseline

NPP (filled bars) in all treatment groups. In control animals (group 1), matched doses of β -FXIIa and NPP produced comparable peak increments of Δ SBP, Δ DBP and Δ HR. Administration of +CAP or +GB alone (groups 2 and 3), or in combination (group 4), affected Δ SBP, Δ DBP and Δ HR maximum responses to NPP and β -FXIIa similarly.

Effects of human NPP and coagulation β -FXIIa on plasma levels of the BK 1-5 metabolite (Figure 5)

After β -FXIIa and NPP were given, there was a twofold increase in plasma BK 1-5 concentrations relative to corresponding baseline values. The values for NPP and β -FXIIa were comparable throughout.

Effects of NPP on plasma catecholamine levels in bioassay rats (Table 2)

Group 1, -GB-CAP ($n=15$): In control animals at peak SBP pressor response to NPP, plasma E levels increased sixfold and the ratio of E to NE increased from approximately 1:1 to 6:1.

Group 2, -GB+CAP ($n=16$): After +CAP, the basal level of E, but not of NE, rose significantly above the corresponding -CAP value. In response to NPP, both E and NE rose substantially (18- and threefold, respectively) and significantly above the corresponding peaks in -CAP rats, raising the E to NE ratios from a baseline of 3:1 to a peak of 15:1.

Group 3, +GB-CAP ($n=14$): With blockade of the cholinergic pathway to the adrenal medulla, the basal E and NE levels fell significantly below control values (group 1). However, at peak SBP, NPP still provoked increases in plasma E (21-fold) and NE (threefold), raising the baseline E to NE ratio from approximately 2:1 to 12:1. These elevations were not significantly higher than the corresponding peak values observed in group 1.

Group 4, +GB+CAP ($n=15$): Under both cholinergic blockade and ACE inhibition, NPP increased plasma E and NE by 48-fold and sevenfold, respectively, raising the E to NE ratio from approximately 2:1 to 17:1. Although +CAP alone (group 2) elevated peak levels of plasma E and NE quite substantially, the addition of GB (group 4) boosted the effects of +CAP even higher (group 4 versus group 2).

TABLE 2

Effect of human new pressor protein (NPP) on plasma catecholamines in bioassay rats

Group 1, -GB-CAP ($n=15$)	E (pg/mL)	NE (pg/mL)
Baseline	180 $\pm$ 30	170 $\pm$ 50
Peak	1120 $\pm$ 320*	190 $\pm$ 90
Δ Peak	940 $\pm$ 300*	20 $\pm$ 4
Group 2, -GB+CAP ($n=16$)		
Baseline	350 $\pm$ 70†	140 $\pm$ 40
Peak	6380 $\pm$ 360*†	430 $\pm$ 100*†
Δ Peak	6030 $\pm$ 350*†	280 $\pm$ 40*†
Group 3, +GB-CAP ($n=14$)		
Baseline	80 $\pm$ 10†	40 $\pm$ 10†
Peak	1680 $\pm$ 280*	140 $\pm$ 30*
Δ Peak	1330 $\pm$ 270*	100 $\pm$ 20*
Group 4, +GB+CAP ($n=15$)		
Baseline	170 $\pm$ 60†‡	70 $\pm$ 20†
Peak	8290 $\pm$ 410*†‡	480 $\pm$ 80*†
Δ Peak	8120 $\pm$ 400*†‡	410 $\pm$ 60*†‡

Comparison of epinephrine (E) and norepinephrine (NE) after injection of human NPP. The values correspond to baseline, peak and Δ peak. Data presented as means $\pm$ SEMs. * $P<0.01$ peak versus corresponding baseline level; † $P<0.01$ for the group receiving captopril (+CAP) versus the corresponding group receiving no captopril (-CAP); ‡ $P<0.01$ for the group with ganglion blockade with pentolinium (+GB) versus the corresponding group with no ganglion blockade (-GB)

DISCUSSION

We previously described several aspects of the potent cardiovascular and sympathoadrenal properties of NPP/ β -FXIIa in bioassay animals (1-6) and have proposed the possible involvement of NPP/ β -FXIIa in hypertensive patients on hemodialysis (15). Such observations suggest that, as a naturally occurring activation product of FXII, NPP/ β -FXIIa could play a potentially significant role in a variety of prevalent pathophysiological conditions that involve inflammation with activation of the blood coagulation system and related enzyme cascades. The present study provides additional confirmation of the co-identity of NPP and β -FXIIa, extends our knowledge of their functional properties and, for the first time, elucidates their complex integrated mechanisms of action.

The cardiovascular actions of NPP/ β -FXIIa depend substantially on sympathoadrenal catecholamines

There is a close relationship between plasma catecholamine concentrations – notably E (Table 2) – and the BP and HR effects shown in Figures 1 to 4. The cardiovascular effects of NPP/ β -FXIIa (Figure 4; groups 2 and 4) are greatly attenuated following acute adrenal medullectomy (1,2) or by adrenergic blockade with phentolamine and propranolol (2), thereby implicating the adrenal medulla. In addition, all of our experimental groups (Table 2) showed increases of plasma catecholamines coincident with the peak SBP response to NPP/ β -FXIIa, suggesting a causal relationship.

Evidence for cholinergic control of basal sympathoadrenal catecholamine release

GB drugs, such as hexamethonium and pentolinium, are well known to interfere with impulse transmission from preganglionic to postganglionic neurons, thereby blocking their stimulation by acetylcholine (24). Adrenal chromaffin cells, which release E and NE into the bloodstream (24), are considered to be postganglionic (25) and therefore predominantly under cholinergic control (26,27). GB with pentolinium (+GB) suppresses cholinergically driven adrenal secretion of E and NE, as observed in the +GB groups (Table 2; groups 2 and 4), which exhibited depressed basal plasma E and NE levels relative to

their corresponding controls (groups 1 and 2), irrespective of +CAP treatment, implicating a cholinergically driven mechanism for basal catecholamine release.

Evidence that +CAP stimulates an 'alternate pathway' for sympathoadrenal catecholamine release

The potent sympathoadrenal effects of NPP are potentiated by +CAP (Table 2; groups 2 and 4), regardless of cholinergic blockade (+GB). Such positive effects by +CAP suggest some 'alternate peptidergic pathway' by which NPP stimulates adrenal catecholamine release. It is well known that CAP inhibits the degradation of peptides regulated by ACE, thereby increasing their bioactivity (7,28). The potentiation of NPP's effects by +CAP could therefore be attributable to the enhancement of a peptidergic mechanism controlled by ACE, independent of +GB. We have shown that ACE-dependent peptides, such as BK (4) and PACAP (3), stimulate adrenal catecholamine release in bioassay animals, especially after +CAP treatment. BK and PACAP, among other ACE-metabolized peptides, could participate in an 'alternate peptidergic pathway' by which NPP stimulates the release of sympathoadrenal catecholamines.

Direct evidence for participation of BK and PACAP in the proposed 'alternate peptidergic pathway'

Exogenous BK (4), as well as PACAP-27 and PACAP-38 (3), duplicate some of the cardiovascular and sympathoadrenal effects of NPP/β-FXIIa in +GB rats, which are also potentiated by +CAP. Administration of the BK B-2 receptor antagonist HOE-140 (4) or the PACAP antagonist PACAP 6-38 (3), attenuate the effects of NPP/β-FXIIa, supporting at least partial mediation by each. The involvement of BK is further supported by the observation of elevated plasma BK 1-5 concentrations (Figure 5), at the peak of the pressor response to NPP/β-FXIIa. BK 1-5 is a stable metabolite of BK used for reliable estimation of its circulating levels (21-23,29), which would be expected to rise in view of the strong association between the coagulation FXIIa and the kallikrein-kinin systems (30,31). Although BK is usually considered to be a vasodilator agent, it can exhibit pressor effects (32) mediated by adrenal catecholamines (33) primarily via BK B-2 receptors, even in pithed animals (34).

How does +GB influence the proposed 'alternate peptidergic pathway'?

As expected, suppression of the cholinergic pathway by +GB depressed basal plasma E and NE concentrations (Table 2; groups 3 and 4 versus groups 1 and 2). Despite the absence of cholinergic stimulation of the adrenal system, the peak response to NPP/β-FXIIa remained comparably elevated in group 3 versus group 1, and even higher in group 4 versus group 2, suggesting that the 'peptidergic pathway' superceded and surpassed the effects of cholinergic control. There appears to be positive synergism between the effects of +GB and +CAP, as though cholinergic suppression by +GB serves to invoke and enhance the peptidergic mechanism, thus potentiating the response to NPP/β-FXIIa (group 4 versus group 2) beyond the level attributable to +CAP alone (group 2 versus group 1).

External evidence of cholinergic and peptidergic mechanisms for sympathoadrenal catecholamine release and their interaction

Using an isolated rat adrenal preparation, Wakade et al (27,35,36) demonstrated the importance of acetylcholine (ACh) for catecholamine release via nicotinic and, to a lesser extent, muscarinic receptors. In addition to ACh, several peptides were coreleased at the medullary synapse that were capable of affecting catecholamine synthesis and secretion (27). One such peptide, PACAP, stimulated chromaffin cell exocytosis by a different mechanism from ACh, especially when the cholinergic system was desensitized or failing (37). Thus, cross-communication appears to exist between ACh and peptide transmitters in controlling catecholamine secretion (38), suggesting cooperation between cholinergic and peptidergic control of the adrenal medulla.

Wakade also showed that high-intensity electrical stimulation (10 Hz to 30 Hz) of the adrenal's splanchnic nerve favoured cholinergically driven catecholamine release, producing modest E to NE ratios of approximately 4:1. Comparable ratios were observed with high concentrations of ACh (10 μM), suggesting that high-intensity electrical stimulation operated primarily through the ACh pathway (27,39). In contrast, much higher E to NE ratios were observed with low-intensity stimulation (0.5 Hz to 3 Hz) and adrenal perfusion with vasoactive intestinal peptides (10 μM; ratio of 7:1) or PACAP (0.1 μM; ratio of 10:1) (39,40). Stimulation of the adrenal system directly with peptide transmitters during low-intensity electrical stimulation of the splanchnic nerve produced high E to NE ratios, demonstrating a predominantly peptidergic mechanism.

NPP/β-FXIIa provokes high catecholamine release and E to NE ratios consonant with a shift to peptidergic control

In control animals (Table 2; group 1), after NPP/β-FXIIa injections, the E to NE ratio was 6:1, influenced substantially by cholinergic mechanisms. With cholinergic blockade (+GB, group 3), the E to NE ratio rose to 12:1, demonstrating enhanced peptidergic influence on the adrenal as a consequence of a reduced cholinergic system. The E to NE ratio increased further to 15:1 (group 2) and 17:1 (group 4) with +CAP, supporting the hypothesis of a shift to an ACE-metabolized peptidergic mechanism for adrenal catecholamine release by NPP/β-FXIIa.

Is the shift to peptidergic control attributable to cholinergic suppression or due to inadequate GB?

A high dose of pentolinium (19.2 mg/kg; refer to the Methods section) was used to ensure effective blockade (41,42). Basal BP dropped in +GB animals to 80/45 mmHg, similar to that observed in +SPT animals (Table 1). Pressor responses to NPP/β-FXIIa in +GB and +SPT animals (group 3) were also comparable (Figure 4), and combining the two treatments (+GB+SPT) did not magnify the effectiveness, suggesting that each had been fully adequate. It appears that our observations in +GB animals were indeed obtained under full GB.

Are NPP/β-FXIIa effects attributable simply to a lowered basal BP?

The lowered basal SBP/DBP values in our +GB animals could have influenced the magnitude of their pressor responses to NPP/β-FXIIa, but our data suggest otherwise. For example, +GB-CAP (group 3) versus +GB+CAP (group 4) animals had comparably lower basal pressures (Table 1) yet their sympathoadrenal responses were remarkably different (Table 2), as were their cardiovascular responses to NPP/β-FXIIa (Figure 1). Such divergent responses, irrespective of basal pressures, were also observed in groups 1 and 2, suggesting that a lowered basal BP alone does solely account for the observed properties of NPP/β-FXIIa.

Unifying theory for the mechanism of action of NPP/β-FXIIa

Supported by our findings (1-6), we propose the following mechanism of action for NPP/β-FXIIa (Figure 6). NPP/β-FXIIa, a naturally occurring product of activation of coagulation FXII (30,31), recruits endogenous peptide mediators to stimulate catecholamine release via an alternate 'peptidergic pathway' to produce potent cardiovascular effects. These peptide mediators probably work in cooperation with the cholinergic system to stimulate catecholamine release, but their relative contribution can increase to the point of dominance when the cholinergic pathway is suppressed. Two peptidic mediators have been implicated to date (BK [4] and PACAP [3]), both of which are known to provoke adrenal catecholamine release (34,39,40,43). Potentiation of NPP/β-FXIIa by ACE inhibition (+CAP) notably under cholinergic blockade (+GB), suggests that an alternate noncholinergic (peptidergic) pathway assumes the dominant role in mediating the sympathoadrenal and cardiovascular effects of NPP/β-FXIIa.

Figure 6 Proposed mechanism of action of the coagulation beta factor XIIa (β -FXIIa)/human new pressor protein (NPP) pathway. ACE Angiotensin converting enzyme; BK Bradykinin; BP Blood pressure; CAP Captopril; FXII Coagulation factor XII; FXIIa Activated coagulation factor XII; α -FXIIa Alpha-fragment of FXIIa; β -FXIIa beta-fragment of FXIIa; GB Ganglion blockade; HMWK High molecular weight kinogen; HR Heart rate Kallikrein; NEP Neutral endopeptidase 24:11; PACAP Pituitary adenylate cyclase-activating polypeptide; PreKall Prekallikrein; SPT Spinal transection

CONCLUSIONS

Our results point to a novel regulatory axis linking an activation product of coagulation FXII to sympathoadrenal release of catecholamines with high plasma E to NE ratios. Elevated levels of coagulation FXIIa occur in common inflammatory and procoagulant pathophysiological conditions (10,13-15,44-46) and could be responsible for otherwise unexplained elevations of adrenomedullary catecholamine levels in mild essential hypertension (47). Pathophysiological activation of such an 'alternate peptidergic pathway' in combination with ACE inhibitor therapy could exacerbate sympathoadrenal catecholamine release and thereby act to oppose the antihypertensive properties of ACE inhibitors. Further investigations are necessary to elucidate these complex integrated mechanisms and their clinical significance.

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