Brain Natriuretic Peptide
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Brain Natriuretic Peptide

PROEFSCHRIFT

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Prof. dr. D.J. Webb (University of Edinburgh, Scotland)
Een hart dat pompt
De hartstocht
Waarmee jij
De dagen vult

Jouw bezigheid
Als bloed
Dat door mijn aderen stroomt

(Daniela Depau, 2003)
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Chapter 1

General introduction
General introduction.

The cardiovascular system is under the control of several hemodynamic and neurohumoral mechanisms. These regulatory mechanisms play a key role in modulating cardiac function, vascular tone, and structure. Although they are essential in vascular homeostasis, they become maladaptive in cardiovascular disease states such as hypertension and heart failure. Except for the autonomic nervous system and the renin-angiotensin-aldosterone system, this concerns the natriuretic peptides. The family of natriuretic peptides consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) of primarily cardiac origin, and c-type natriuretic peptide (CNP) of endothelial origin. Most research has focused on ANP, which was the first natriuretic peptide to be identified. Recently, however, many efforts have been directed towards BNP. In humans this peptide acts as a marker for left ventricular hypertrophy and cardiac failure. However, the (patho)physiological significance of BNP in cardiovascular homeostasis in man is not yet clear. Even though BNP has been successfully applied in the treatment of congestive heart failure, it remains elusive by which mechanisms this compound affects the vascular system and renal function. This lack of knowledge formed the impetus for this thesis. The overall objective of this thesis consist of two questions:

1. What are the hemodynamic effects of BNP on different target organs in humans?
2. What are the underlying mechanisms of action of BNP?

The hemodynamic and renal effects of low-dose BNP infusion in normal man are described in chapter 2, while chapter 3 illustrates the local renal effects of BNP in hypertensive patients. The study in chapter 4 discusses endothelin-1 as a possible mediator of the BNP-induced renal effects. In chapter 5 the local effects of BNP on forearm vasculature are compared to those of ANP. Mechanisms of action of BNP that are involved in the effects on the forearm vasculature are described in chapter 6. Finally, chapter 7 gives an overview of (recent) knowledge about BNP in healthy subjects and patients with hypertension and/or heart failure and the results of this thesis are discussed in the light of current literature. Chapter 8 and 9 concisely summarize the contents of this thesis.
Chapter 2

Hemodynamic and renal effects of low-dose Brain Natriuretic Peptide infusion in man

K van der Zander, AJHM Houben, L Hofstra, AA Kroon, PW de Leeuw
Abstract

The present study was designed to investigate the effects of low-dose brain natriuretic peptide (BNP) infusion on central, renal hemodynamics and microvascular hemodynamics in healthy subjects. To this end, we infused BNP or placebo for 1 hour, on two separate days, in 12 healthy subjects. Nailfold and conjunctival capillary density, finger skin microvascular blood flow (SBF), cardiac output, effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were studied before and after infusion. Blood pressure and heart rate (HR) were monitored as well. Compared to placebo, BNP significantly decreased stroke volume with a tendency to decrease cardiac output. BNP decreased MAP and increased HR increased in sitting, but not in supine position. Natriuresis, diuresis, GFR, filtration fraction, and filtered load of sodium increased during BNP relative to placebo, while ERPF did not change. BNP did not affect the microvascular capillary density of conjunctiva and skin, SBF, total skin oxygen capacity and post-occlusive recruitment. The present results suggest that BNP has predominantly central and renal hemodynamic effects, while it does not influence peripheral microcirculation, at least in skin and conjunctiva.
Introduction

Despite many investigations, the hemodynamic effects of brain natriuretic peptide (BNP) remain elusive. Whereas cardiac output (CO) and heart rate (HR) increased during BNP infusion in one study, these variables did not change in two others. Although variations in the cardiovascular effects of BNP could be due to differences in BNP levels reached during the experiments, it is equally possible that changes in cardiac output depend upon alterations in preload and, hence, upon differential effects of BNP on arteriolar and venular tone. Unfortunately, in man no information is available with respect to the main side of action of BNP in the vasculature. Therefore, the aim of our study was to investigate the effects of BNP on both central and peripheral hemodynamics in normal subjects. To this end, we performed a double blind randomised placebo-controlled crossover study with measurements of blood pressure, heart rate, cardiac output, renal hemodynamics, and various microcirculatory elements of the conjunctiva and skin before and after infusion of either placebo or BNP. In addition, we evaluated whether intrarenal forces could explain the enhanced natriuresis occurring during BNP administration. Because the pathophysiological role of BNP becomes particularly evident in older patients we selected for this study only individuals above age 50 years.

Materials and methods

Subjects

Experiments were performed in 12 healthy volunteers. During the week prior to the measurements, all subjects adhered to a 175 mmol Na\(^+\)-containing diet so as to minimize variations in results due to salt intake. Compliance with the diet was checked by measuring sodium and creatinine output in 24-hour urine collections obtained during the last 24 hours before the first experimental day. None of the subjects used any medication (including non-steroidal anti-inflammatory drugs) during the two weeks prior to the measurements. In addition, they had to refrain from smoking and drinking caffeine or alcohol containing beverages for at least 12 hours before the experiments, which started at 8:30 a.m. after an overnight fast. The Medical Ethics Committee of the Maastricht University Hospital approved the study, and all participants gave written informed consent. The investigations conformed to the principles outlined in the Declaration of Helsinki.
Experimental design

All volunteers were studied on two separate occasions (at least two days apart), during which they received in random order (double blind) an i.v. infusion of either BNP or vehicle (glucose 5%). Experiments were performed in a quiet, temperature-controlled room (mean temperature 24.2±0.3°C). Precautions were taken to minimize external disturbances. Except during the microcirculatory measurements (which had to be performed in sitting position), subjects remained supine throughout the experiments. A 20-gauge catheter was inserted into the antecubital vein of both arms. One was connected to a 3-way tap for infusion of BNP (Clinalfa, Ethifarma Nederland BV, The Netherlands) and para-amino hippurate (PAH)/inulin (for measuring renal hemodynamics), while the other was used for blood sampling. To ensure adequate diuresis, subjects consumed 200 mL of water every hour until the last blood samples had been drawn. At t=0 min the PAH/inulin infusion was started. Between t=0 and t=60 min baseline measurements of the conjunctiva and nailfold microcirculation, skin blood flow, and total skin oxygen capacity were obtained. At t=60 min an echocardiogram was taken. At t=120 min the intravenous infusion of either BNP (4 pmol/kg/min) or placebo (glucose 5%) was started. At t=180 min (i.e. after one hour of BNP or placebo infusion), a second echocardiogram was performed, followed by a second set of microvascular measurements. Blood pressure and heart rate (HR) were measured before and after the microvascular measurements in sitting position, and at 10-minute intervals during the infusion in supine position. Blood samples were drawn at t=0, t=120, and t=180 min for PAH and inulin and at t=120 and t=180 min for determination of cGMP and BNP. Urine samples for measurement of sodium and potassium were collected at t=60, t=120, and t=180 min (immediately after blood sampling).

Measurements

Systemic hemodynamics

Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and HR were measured by a semiautomatic oscillometric device (Dinamap Vital Signs Monitor 1846, Critikon). CO was measured by echocardiography and total peripheral resistance (TPR) was calculated as (MAP / CO) * 80 and expressed in dyne · s/cm².

Microcirculatory measurements

The microcirculation of the lateral part of the bulbar conjunctiva of the right eye was studied with a custom build horizontal microscope, as described before. For microvascular density measurements, recordings
were made on videotape with a standard achromatic objective 2.5x (numeric aperture (N.A.): 0.10). Arterioles, capillaries and venules were classified in several videoframes, using image analysis software (OPTIMAS version 5.0, Breda, The Netherlands). As a measure of density the total length of each microvascular class per square millimetre of conjunctiva was determined and averaged.

Measurement of nailfold capillary density was also performed as described earlier. For the capillary density measurements, recordings were made a few millimetres proximal to the terminal row of capillaries. Baseline skin capillary density was defined as the amount of erythrocyte-filled capillaries in one videoscreen (1.6 mm² of skin). The recruitment of functionally available capillaries was defined as the increase in the number of erythrocyte-filled capillaries after 4 min of arterial occlusion (by cuff inflation of 200 mmHg at the wrist).

Skin blood flow (SBF), which predominantly reflects thermoregulatory flow, was determined simultaneously with nailfold capillary density using laser-Doppler fluxmetry (Periflux PF3; Perimed, Järfalla, Sweden), with probe PF 308, wide band (12 kHz) mode, and time constant 0.2 s. Total skin oxygen capacity, a measure of nutritive blood flow, was determined using transcutaneous oxygen tension measurements (TePo2, Radiometer), with the probe heated to 44°C. Probes were placed on the dorsum of the interphalange of the finger and the hand between digit IV and V, respectively, of the same hand as nailfold capillary density was measured in. Flux values are expressed as arbitrary perfusion units, calibrated against an external standard. Total skin oxygen capacity is defined in mmHg. SBF and total skin oxygen capacity were measured before and during reactive hyperemia following 4 min of arterial occlusion (200 mmHg).

Renal function

Renal hemodynamics, i.e., effective renal plasma flow (ERPF) and glomerular filtration rate (GFR), were measured as the clearance of PAH (MSD, West Point, PA, USA) and inulin (Inutest, Laevosan Gesellschaft, Linz, Austria), respectively, during continuous infusion of these substances. Both GFR and ERPF were corrected for body surface area and expressed as mL/(min · 1.73 m²). Effective renal blood flow (ERBF) was calculated by the following formula: ERPF/(1-hematocrit). Filtration Fraction (FF) was calculated as GFR/ERPF. Renal vascular resistance (RVR) was calculated according to the formula: (MAP/ERBF) * 80 000 and expressed in dyne · s/cm². Renal Fraction (RF) was calculated as (ERBF/CO) * 100%.

Filtered Load (FLNa) of sodium is calculated as GFR * 60 * [Na⁺]plasma and expressed in mmol/hr. Fractional Tubular Reabsorption of sodium (FTRNa) is calculated as ( (FLNa - UNaV) / FLNa ) * 100%, where UNa and V are the urinary sodium concentration and urinary volume respectively.
Assay methods

PAH and insulin levels were measured by means of a spectrophotometer. BNP and cGMP levels were measured by means of a competitive protein-binding RIA (Peninsula Laboratories Inc. RIK 9086 and IBL Hamburg RE 29071, respectively). Prior to assay, plasma samples of BNP were acidified and extracted using a SEP-Pak C18 column (Waters-Millipore). In our hands, the intra- and interassay variability of all assays were <10%. All samples from the same individual were assayed in a single run.

Statistics

Data are presented as medians with interquartile ranges (IQR). The Wilcoxon paired sign test for paired analysis (both within one visit and between the BNP and placebo infusion) and Friedman's test for analysis of multiple related measurements of MAP and HR (within one visit) were used. P values below 0.05 denote statistical significance. Based on previous experiments we calculated that this study is able to demonstrate in 12 experimental subjects a 10% difference in any of the test variables with a power of 85%.

Results

Baseline clinical characteristics of the study participants are summarised in Table 2.1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (male/female)</td>
<td>12 (10/2)</td>
</tr>
<tr>
<td>Age, year</td>
<td>61 (57-63)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.0 (24.0-27.1)</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>124 (120-128)</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>82 (81-83)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>61 (60-68)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mmol/24h</td>
<td>151 (141-206)</td>
</tr>
</tbody>
</table>

(Blood pressure is the mean of 3 measurements by sphygmomanometer)
BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HR, Heart rate.

Plasma levels of BNP and cGMP

At baseline, BNP plasma levels did not differ between visits (Table 2.2). BNP infusion significantly increased plasma levels of this peptide to 191
(172-367) pg/mL (p<0.01), while placebo infusion had no effect on BNP levels (21 [11-68] pg/mL after infusion). In parallel with the increase in BNP, plasma levels of cGMP also significantly increased to 19.5 (15.6-25.6) pmol/mL (p<0.01) after BNP infusion, while placebo infusion did not change levels of cGMP [6.6 (5.7-7.5) pmol/mL after infusion]. No differences between cGMP values at baseline were observed (Table 2.2).

Table 2.2 Baseline data of all variables [supine] before placebo or BNP infusion.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>BNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP, pg/mL</td>
<td>23 [12-34]</td>
<td>15 [11-41]</td>
</tr>
<tr>
<td>cGMP, pmol/mL</td>
<td>6.0 [5.1-8.0]</td>
<td>6.1 [4.5-7.7]</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>97 [87-100]</td>
<td>97 [91-101]</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>54 [50-59]</td>
<td>58 [55-59]</td>
</tr>
<tr>
<td>SV, mL</td>
<td>88 [80-97]</td>
<td>84 [63-92]</td>
</tr>
<tr>
<td>CO, L</td>
<td>4.9 [4.1-6.3]</td>
<td>4.8 [4.1-6.0]</td>
</tr>
<tr>
<td>TPR, dyne · s/cm²</td>
<td>1619 [1258-1930]</td>
<td>1580 [1272-1933]</td>
</tr>
<tr>
<td>ERPF, mL/min · 1.73 m²</td>
<td>386 [315-488]</td>
<td>413 [334-439]</td>
</tr>
<tr>
<td>ERBF, mL/min · 1.73 m²</td>
<td>612 [546-871]</td>
<td>745 [566-759]</td>
</tr>
<tr>
<td>GFR, mL/min · 1.73 m²</td>
<td>99 [91-115]</td>
<td>104 [90-115]</td>
</tr>
<tr>
<td>FF, %</td>
<td>26 [24-30]</td>
<td>26 [23-29]</td>
</tr>
<tr>
<td>RVR, dyne · s/cm²</td>
<td>10622 [8164-12371]</td>
<td>9409 [7182-13309]</td>
</tr>
<tr>
<td>RF, %</td>
<td>16 [10-18]</td>
<td>17 [11-20]</td>
</tr>
<tr>
<td>U₁₀₅V, mmol/hr</td>
<td>9.0 [7.3-11.8]</td>
<td>6.0 [5.7-9.6] *</td>
</tr>
<tr>
<td>V₅, mL/hr</td>
<td>105 [66-230]</td>
<td>70 [41-154]</td>
</tr>
<tr>
<td>FTP₅, %</td>
<td>99.1 [98.9-99.3]</td>
<td>99.3 [98.8-99.5] *</td>
</tr>
</tbody>
</table>

Data are expressed as medians and IQR. * P<0.05 versus placebo.

Systemic hemodynamic effects

Systemic hemodynamic in supine position

At baseline, no differences in blood pressure or heart rate were observed between BNP and placebo experiments (Table 2.2). MAP was not altered by infusion of either BNP or placebo (Figure 2.1). During BNP infusion supine HR tended to rise but differences from baseline just failed to reach statistical significance (p=0.058); moreover, HR responses did not differ between the two infusion experiments (Figure 2.1; p=0.686). Baseline values of stroke volume (SV) and CO did not differ either (Table 2.2). SV significantly decreased after BNP infusion (to 73 [66-83] mL; p=0.007) while it did not change during placebo (83 [76-93] mL; p=0.594). This difference between the effects of BNP and placebo was statistically significant (p=0.015; Figure 2.2). CO significantly decreased to 4.3 L [3.7-5.2] during BNP infusion (p=0.013), while placebo did not induce any significant change (to 4.6 [4.2-5.2] mL; p=0.182). Expressed
as percent change, BNP had no significant effect on CO compared to placebo (p=0.082; Figure 2.2). TPR was not influenced by infusion of either BNP (1.880 (1.453-2.049) dyne • s/cm²) or placebo (1.755 (1.426-2.004) dyne • s/cm²).

Figure 2.1 Suprare heart rate (HR, panel A) and mean arterial blood pressure (MAP, panel B) at baseline and every 10 minutes during 1 hour of placebo or BNP infusion. Data are presented as median and interquartile ranges. No significant changes were observed.

Influence of posture on BNP effects: MAP and HR in sitting position
Postural changes during BNP infusion were not tolerated well by four subjects. Two of these became markedly hypotensive in sitting position 15 min after completion of the BNP infusion and were unconscious for a few minutes. The other subjects “only” appeared pale and experienced dizziness and sweating in sitting position at the same moment during the protocol.
At baseline, sitting MAP and HR did not differ between BNP and placebo experiments. BNP infusion decreased sitting MAP from 92 (86-103) mmHg to 80 (72-93) mmHg (p=0.05). This BNP-induced fall in pressure was significantly greater than that during placebo (p=0.028). HR increased significantly after BNP infusion (from 59 (56-63) beats/min to...
68 (60-78) beats/min; p=0.011) but there was no difference in percent change in HR compared to placebo infusion (p=0.239).

![Bar graph showing % change in cardiac hemodynamics for SV and CO between Placebo and BNP groups.](image)

**Figure 2.2** Cardiac hemodynamic responses of stroke volume (SV) and cardiac output (CO) to intravenous infusion of placebo or BNP, expressed as percent change. Data are presented as median and interquartile ranges. *p<0.02.

![Bar graph showing % change in renal hemodynamics for ERPF, GFR, and FF between Placebo and BNP groups.](image)

**Figure 2.3** Renal hemodynamic responses of renal plasma flow (ERPF), glomerular filtration rate (GFR) and filtration fraction (FF) to intravenous infusion of placebo or BNP, expressed as percent change. Data are presented as median and interquartile ranges. *p<0.03.
Renal effects

Renal hemodynamics

Baseline renal hemodynamics did not differ between BNP and placebo (Table 2.2). BNP significantly increased GFR after BNP infusion compared to placebo (p=0.007 BNP vs. placebo, Figure 2.3), while ERPF did not change during either infusion. FF increased during BNP infusion to 29 (26-31) %, while it tended to decrease during placebo infusion (to 25 (21-28) %; p=0.022 BNP vs. placebo, Figure 2.3). BNP tended to increase RVR to 11 526 (7 612-12 916) dyne · s/cm², but the difference just failed to reach statistical significance (p=0.063). However, there was no difference in percent change compared to placebo infusion (p=0.465). RF increased during placebo infusion to 18 (12-20) %; p=0.033, while no difference was observed in RF responses between placebo and BNP infusion (p=0.674).

Natriuresis and diuresis

At baseline, UₜₙV was slightly lower and FTRₜₙ slightly higher on the BNP than on the placebo day (Table 2.2). Compared to placebo infusion, BNP infusion resulted in a significant natriuresis and diuresis. Urinary sodium excretion in a one-hour urine collection increased to 24.1 (15.9-28.2) mmol during BNP (p=0.002), while it did not change during placebo infusion (10.3 (7.3-13.0) mmol/h after infusion; p=0.308). Urinary volume of the one-hour urine collection significantly increased both after BNP (to 408 (360-584) ml) and after placebo infusion (to 225 (176-314) ml) as a result of water suppletion during the renal clearance study protocol. However, the increase in urinary volume after BNP infusion was significantly greater than after placebo infusion (p=0.002). FLAN increased significantly from 855 (753-939) mmol/hr to 894 (803-983) mmol/hr during BNP infusion, while placebo infusion did not influence FLAN. Furthermore, BNP significantly decreased FTRₜₙ (to 97.4 (97.1-98.2) %) as compared to placebo infusion (p=0.005).

Microcirculation

Skin blood flow

The effects of BNP on both basal and hyperaemic peak skin (thermoregulatory) blood flow are shown in Table 2.3. As compared to placebo, BNP did not change any of the variables. Also, time to peak and duration of hyperaemia were not influenced by infusion of either BNP or placebo. Furthermore, no differences in total skin oxygen capacity at 44 °C were observed (Table 2.3).
**Nailfold microcirculation**

Although small changes in capillary density or post-occlusive recruitment were observed during either placebo or BNP infusion, overall no significant changes between the two experiments could be observed (Table 2.3).

Table 2.3 Finger skin capillary density, post-occlusive (4 min) reactive capillary recruitment and SBF, and TcPo2 (44°C) before (baseline) and percentage change after 1 h BNP or placebo infusion (i.v.)

<table>
<thead>
<tr>
<th></th>
<th>Placebo Baseline</th>
<th>%-Change</th>
<th>BNP Baseline</th>
<th>%-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary density</td>
<td>96 (84-110)</td>
<td>4 (0-13)</td>
<td>92 (79-107)</td>
<td>12* (6-21)</td>
</tr>
<tr>
<td>number/1.6 mm² skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recruitment</td>
<td>16 (9-18)</td>
<td>-59* (-87-7)</td>
<td>9 (6-15)</td>
<td>-21 (-79-38)</td>
</tr>
<tr>
<td>number/1.6 mm² skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest flux, pu</td>
<td>13 (10-19)</td>
<td>-25* (-45-1)</td>
<td>13 (10-23)</td>
<td>-14 (-30-15)</td>
</tr>
<tr>
<td>Peak flux, pu</td>
<td>47 (39-85)</td>
<td>-22 (-34-17)</td>
<td>58 (36-74)</td>
<td>-8 (-20-18)</td>
</tr>
<tr>
<td>TcPo2, mmHg</td>
<td>47 (25-61)</td>
<td>-3 (-34-9)</td>
<td>44 (35-63)</td>
<td>-14 (-25-3)</td>
</tr>
</tbody>
</table>

Data are expressed as medians and IQR. * p<0.05 versus baseline. No significant differences were observed between placebo and BNP.

**Conjunctival microcirculation**

Arteriolar, capillary, and venular density did not change significantly either during BNP or during placebo infusion (Table 2.4).

Table 2.4 Conjunctival microvascular densities (mm/mm²) at baseline and percent change in baseline values after 1 h BNP or placebo infusion (i.v.)

<table>
<thead>
<tr>
<th></th>
<th>Placebo Baseline</th>
<th>%-Change</th>
<th>BNP Baseline</th>
<th>%-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteriolar density</td>
<td>0.8 (0.7-1.0)</td>
<td>3 (-20-29)</td>
<td>0.9 (0.8-1.1)</td>
<td>6 (-33-36)</td>
</tr>
<tr>
<td>Capillary density</td>
<td>1.9 (1.0-2.6)</td>
<td>10 (0-59)</td>
<td>1.8 (0.8-3.0)</td>
<td>15 (-7-32)</td>
</tr>
<tr>
<td>Venular density</td>
<td>3.1 (2.8-3.8)</td>
<td>-10 (-21-34)</td>
<td>3.0 (2.4-3.7)</td>
<td>0 (-14-15)</td>
</tr>
</tbody>
</table>

Data are expressed as medians and IQR. No significant changes were observed.

**Discussion**

The results of the present study suggest that low-dose BNP, when administered systemically, has predominantly central and renal hemodynamic effects, while it does not influence peripheral microcirculation, at least in skin and conjunctiva.
In supine position, BNP infusion significantly reduced SV and tended to decrease CO. However, MAP, TPR and HR did not change significantly. Other investigators who reached similar plasma levels of BNP as we did, also observed decreases in SV without changes in CO and MAP, whereas HR only increased after infusion of higher doses of BNP. These observations suggest that BNP primarily reduces preload, possibly by lowering venous return. This hypothesis is supported by our observations on the hemodynamic pattern in sitting position. Indeed, when subjects rose, they experienced orthostatic symptoms, but we also found a significant fall in MAP and CO. At the same time, HR increased rapidly, probably due to activation of the baroreceptor reflex. In literature no such influence of body position on BNP effects has been described yet.

To the best of our knowledge, our study is the first to report that low-dose infusion of BNP has no effects on skin and conjunctival microcirculation in humans. In all likelihood, this lack of effect is not due to methodological problems such as large variability of the measurements, since with the same set-up we were able to demonstrate microvascular effects of atrial natriuretic peptide (ANP). In that study ANP caused vasoconstriction of the microcirculation, mainly on the venular side. When we combine our observations on the systemic and the peripheral vasculature, it would seem that the most probable site of action for BNP is the venous system where it may increase the ‘unstressed’ volume. This would also explain, at least in part, the beneficial effects of this peptide in patients with congestive heart failure. Further studies with measurements of cardiac filling pressures and venous compliance are necessary to confirm or refute this hypothesis.

Of particular interest are the effects of BNP on the kidney. In previous studies, RPF has been found to decrease, to increase, or to remain unchanged during infusion of BNP. However, these studies are difficult to compare with the present one because of differences in design, doses of BNP, and infusion time. For instance, concurrent changes in GFR and RPF only occurred when the dose of BNP exceeded 2pmol/kg/min. With lower doses of BNP, La Villa et al. observed a natriuretic effect of BNP in the absence of changes in RPF and GFR. Our data are in line with the latter observations, even though the dose of BNP that we employed was similar to that in the studies of Jensen et al. and La Villa et al. However, Jensen et al. did not compare placebo and BNP in the same subjects, which may have introduced bias. The difference between our findings and those of La Villa et al. can probably be explained by the small number of (younger) subjects and the lower salt intake in their study as compared to ours. Although RVR tended to increase during BNP infusion, there were no differences in RVR or RF as compared to placebo. Thus, also in one of the major target organs for BNP, the peptide did not markedly influence arteriolar tone.

Besides the expected increase in plasma cGMP, urinary sodium excretion, and urinary volume, we observed a significant increase in
GFR, FF and in filtered load of sodium during BNP. This suggests that BNP has a direct effect on post-glomerular vessels causing vasoconstriction and a rise in FF. Although our results do not allow to draw definite conclusions, they are compatible, at least, with the hypothesis that this vasoconstriction occurs at the level of the peritubular vessels rather than at the level of the efferent arterioles. Indeed, a rise in FF due to increased efferent arteriolar resistance would tend to enhance proximal tubular reabsorption of sodium. In fact, others have demonstrated that proximal reabsorption of sodium may be reduced by BNP\textsuperscript{10,11}, which one would expect if the site of increased resistance is located further down the nephron and intrarenal physical factors raise peritubular hydrostatic pressure.

Although we did not perform a head-to-head comparison of BNP and ANP in this study, data of a previous study from our laboratory suggest that both peptides have not only different effects on microcirculatory, but also on central and renal hemodynamics. In that study we found that ANP had no effect on MAP and HR, even in sitting position.\textsuperscript{5} Furthermore, in that study ANP infusion decreased GFR and RPF and increased RVR to stimulate natriuresis and diuresis.\textsuperscript{5} Finally, ANP caused venular vasoconstriction in the microcirculation. Thus, BNP and ANP seem to have differential actions on the vascular system.

In conclusion, this study suggests that low-dose intravenous infusion of BNP in healthy subjects has predominantly central and renal hemodynamic effects, while it does not influence peripheral microcirculation.
References
Chapter 3

Does Brain Natriuretic Peptide have a direct renal effect in human hypertensives?

K van der Zander, AJHM Houben, AA Kroon, TKA Wierema, MJMJ Fuss-Lejeune, D Koster, and PW de Leeuw
Abstract

Systemic infusion of brain natriuretic peptide (BNP) stimulates natriuresis and diuresis, but has variable effects on the renal vasculature. In this study we investigated whether BNP has any direct effects on the kidney in hypertensive patients. Three stepwise increasing doses of BNP (60, 120, and 180 pmol/min) or placebo were infused into the renal artery of 26 hypertensive patients. Renal blood flow was determined using the $^{133}$Xenon washout technique. Before and after infusion of BNP, arterial and venous blood samples were taken for cGMP, renin, and creatinine concentration. Intra-arterial blood pressure and heart rate were monitored continuously. Intrarenal BNP infusion did not induce significant changes in renal blood flow, despite increases in circulating levels of cGMP. The latter, however, was not associated with changes in the cGMP gradient across the kidney. In addition, we did not find any BNP-related changes in the secretion of active renin and in creatinine extraction. At the highest dose heart rate increased after BNP infusion without a change in mean intra-arterial blood pressure. In conclusion, this study suggests that, at least in hypertensives, BNP has no direct intrarenal hemodynamic effects and that the rise in circulating cGMP without changes in net renal extraction of this second messenger is related to a primary extrarenal target of BNP.
Introduction

Systemic infusion of brain natriuretic peptide (BNP) stimulates natriuresis and diuresis\textsuperscript{1-6}, and inhibits plasma renin activity (PRA)\textsuperscript{1-3,7,8}, but has variable effects on the renal vasculature. Although most, but not all, studies in healthy humans reported that BNP infusion increases glomerular filtration rate (GFR)\textsuperscript{1,5,7}, renal plasma flow (RPF) has been found to decrease\textsuperscript{1,7}, to increase\textsuperscript{5}, or to remain unchanged.\textsuperscript{3,8} Variations in the renovascular effects of BNP could be related to differences in BNP levels reached during the experiments, but it is equally possible that the renal changes are, in part, secondary to systemic effects. Indeed, BNP not only acts on the kidney, but also affects blood pressure, heart rate, cardiac output, and systemic vascular resistance.\textsuperscript{2,3,5,6,8,9} In those studies where changes in RPF are reported, it is important to keep in mind that such alterations may simply be due to concurrent changes in cardiac output. If, for instance, renal fraction (that is the proportion of cardiac output perfusing the kidneys) remains unaltered during systemic BNP infusion, it is unlikely that the peptide has exerted a direct effect on the renal vasculature. If, on the other hand, renal fraction increases, relative renal vasodilatation must have occurred. Unfortunately, in most studies this has not been taken into account.

Local administration of BNP in a regional vascular bed in amounts that will not have systemic effects allows for assessing whether BNP has any direct effects on certain parts of the circulation. With this approach others and we have demonstrated previously that BNP induces a dose-dependent vasodilatation in forearm vasculature.\textsuperscript{10,11} In the present study, we investigated whether BNP has any direct effects on the renal circulation. To this end, we infused BNP into the renal artery of hypertensive patients who were scheduled for renal angiography. Before and during the infusion we measured renal blood flow, renin release and the extraction of creatinine, the latter being taken as a marker of glomerular filtration. We also measured concentrations of cGMP, since this second messenger may reflect BNP's activity.

Methods

Subjects

This study was performed in 26 hypertensive patients in whom renal artery stenosis (RAS) was suspected on the basis of one or more of the following criteria: treatment-resistant hypertension despite the use of at least two adequately dosed antihypertensive agents, overt peripheral
vascular disease, the presence of an abdominal bruit or an increase in serum creatinine during angiotensin-converting enzyme inhibitor treatment. Antihypertensive medication, if any, was discontinued for three weeks before the measurements. Before hospital admission, patients were randomly allocated to a placebo group and a BNP group. Because the effects of BNP are dependent on sodium intake, we instructed half of the patients from the BNP group to follow a salt-restricted diet (55 mmol of sodium/day) and the other half to adhere to a high-salt diet containing 220 mmol of sodium/day during the last week before the study. Compliance with the diet was checked by measuring sodium and creatinine output in 24-hour urine collections obtained during the last day before angiography. Patients were also instructed to refrain from smoking and drinking caffeine or alcohol containing beverages for at least 48 hours before the investigations.

The Medical Ethics Committee of the Maastricht University Hospital approved the study, and all participants gave written informed consent. The investigations conformed to the principles outlined in the Declaration of Helsinki.\textsuperscript{12}

Experimental design

Experiments were performed in the angiography suite of the Department of Radiology, which is equipped with an x-ray system and a gamma camera. After selective catheterisation of the renal artery and vein and before any administration of contrast material, blood samples were drawn simultaneously from the renal artery and both renal veins for determination of active plasma renin concentration (APRC), BNP, cGMP, and creatinine levels. Subsequently, mean renal blood flow (MRBF) was measured, first in the left kidney and then in the right one, by means of the \textsuperscript{133}Xenon washout technique as described earlier.\textsuperscript{13,14} Next, BNP in incremental doses of 60, 120, and 180 pmol/min or placebo (glucose 5\%) was infused into the right renal artery. Each dose was continued for 10 minutes. MRBF was measured at the end of each dosing interval. At the end of the highest dose of BNP, blood samples for determination of APRC, BNP, cGMP, and creatinine levels were drawn again from the right renal vein as well as from the femoral artery. The latter was necessary to avoid contamination of blood by BNP from the infusion line. Blood samples were spun immediately and plasma was stored at a temperature of \textdegree{}80\C until assay. Heart rate (HR) and intra-arterial blood pressure were monitored continuously during each MRBF measurement. Angiography was performed only after all measurements had been completed.

Assay methods

APRC was measured by the IRMA method (Nichols Institute Diagnostics, Wijchen, The Netherlands). BNP and cGMP levels were measured by
means of a competitive protein-binding RIA (Peninsula Laboratories Inc. RIK 9086, and IBL Hamburg RE 29071, respectively). Prior to assay, plasma samples of BNP were acidified and extracted using a SEP-Pak C18 column (Waters-Millipore). In our hands, the intra- and interassay variability of all assays was <10%. The antisera for BNP did not crossreact with the other peptides. All samples from the same subject were assayed in a single run.

Calculations and statistics

The effects of BNP on MRBF were expressed as the integrated vascular response (IVR), defined as the area under the percent change curve and expressed in units (percent change x time). A positive IVR indicates an increase in MRBF (i.e. vasodilatation), whereas a negative IVR denotes a decrease in MRBF (vasoconstriction). Net renal BNP, cGMP, and renin production or extraction was calculated as (venous concentration-arterial concentration) x MRBF. Fractional creatinine extraction was calculated as (arterial concentration-venous concentration) / arterial concentration of creatinine.

Non-parametric statistics were used for analysis. Within-group comparisons were performed using Friedman’s two-way analysis of variance. Between-group analyses were performed using Kruskal-Wallis (one-way ANOVA) tests. Data are presented as medians with interquartile ranges (IQR) unless indicated otherwise. P values below 0.05 denote statistical significance. The Xenon-washout technique provides accurate estimates of renal blood flow and, in our hands, has a variability of 8% for repeated measurements. Therefore, this study is able to demonstrate a 10% difference in MRBF in 10 control and 16 experimental subjects with a power of 85%.

Results

Baseline clinical characteristics of the study participants are summarised in Table 3.1. Although eight patients exhibited some degree of renal artery stenosis, in none of them hemodynamically significant lesions existed.
Table 3.1 Characteristics of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo</th>
<th>BNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (male/female)</td>
<td>10 (6/4)</td>
<td>16 (11/5)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>56 (37-67)</td>
<td>53 (46-68)</td>
</tr>
<tr>
<td>Diagnosis, EH/RAS</td>
<td>10/4</td>
<td>12/4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.7 (22.6-28.0)</td>
<td>25.3 (23.3-27.2)</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>136 (120-144)</td>
<td>140 (123-149)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>77 (66-85)</td>
<td>70 (60-75)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mmol/24h</td>
<td>65 (41-80)</td>
<td>68 (48-83)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS: 204 (161-244)</td>
</tr>
</tbody>
</table>

Data are presented as median and interquartile ranges.
LS = low salt diet. HS = high salt diet

Effect of BNP or placebo infusion on renal blood flow

At baseline, mean renal blood flow (MRBF) did not differ significantly between the placebo and the BNP group (p=0.262; Table 3.2). Likewise, no differences in flow could be detected between patients on a low-salt diet and those on a high-salt diet. Responses did not differ either between kidneys with or without renal artery stenosis. Infusion of placebo did not induce significant changes in MRBF (p=0.976; Table 3.2). However, MRBF was not altered by BNP either (p=0.204; Table 3.2). The latter was true both in the low and in the high-salt group (p=0.308, and p=0.366 respectively). Furthermore, changes in IVR during BNP infusion (1.8 [-11.3-37.0]) did not differ significantly from zero (p=0.234). In addition, there was no difference in IVR among the BNP and placebo group (p=0.856). No relation was seen between IVR and baseline renal flow.

Table 3.2 Absolute flow (mL/100g/min) at baseline and after BNP (or placebo) infusion

<table>
<thead>
<tr>
<th>Infusion of</th>
<th>baseline</th>
<th>60 pmol/min</th>
<th>120 pmol/min</th>
<th>180 pmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>241 (163-290)</td>
<td>233 (156-297)</td>
<td>263 (162-314)</td>
<td>251 (172-305)</td>
</tr>
<tr>
<td>BNP</td>
<td>166 (111-248)</td>
<td>190 (137-236)</td>
<td>176 (166-260)</td>
<td>184 (137-206)</td>
</tr>
</tbody>
</table>

Plasma levels of BNP, cGMP, renin and creatinine

Placebo infusion did not alter any of the measured variables or their gradients across the kidney. Intrarenal BNP infusion not only caused the expected increase in venous levels of this peptide, but also enhanced arterial BNP levels (both p<0.01; Table 3.3). Parallel with the increase in plasma BNP, we observed a rise in cGMP, both in arterial and in venous renal blood samples (both p<0.01). The net BNP and cGMP gradients across the kidney, however, did not change during BNP infusion (p=0.182 and p=0.875, respectively). Moreover, BNP infusion did not
induce significant changes in net APRC production and creatinine extraction (Figure 3.1). Fractional creatinine extraction did not change either (Table 3.3). When the placebo and the BNP group were compared, no differences were observed in APRC production or (fractional) creatinine extraction between both groups.

Table 3.3 Plasma levels of BNP, cGMP, APRC, and creatinine at baseline and after BNP infusion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th></th>
<th>After BNP infusion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arterial</td>
<td>Venous</td>
<td>Arterial</td>
<td>Venous</td>
</tr>
<tr>
<td>BNP, pg/mL</td>
<td>83.6</td>
<td>82.1</td>
<td>510.8*</td>
<td>577.3*</td>
</tr>
<tr>
<td>cGMP, pmol/mL</td>
<td>6.8</td>
<td>5.6</td>
<td>10.3*</td>
<td>7.9*</td>
</tr>
<tr>
<td>APRC, μU/mL</td>
<td>19.0</td>
<td>26.5</td>
<td>26.9</td>
<td>26.4</td>
</tr>
<tr>
<td>Creatinine, mmol/mL</td>
<td>79.5</td>
<td>70.5</td>
<td>78.5</td>
<td>68.0</td>
</tr>
<tr>
<td>fractional creatinine extraction</td>
<td>0.20 [0.13-0.26]</td>
<td>0.20 [0.12-0.26]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.01 versus baseline.

Figure 3.1 Net renal renin (panel A) and creatinine (panel B) production/extraction at baseline and after placebo or BNP infusion. Data are presented as median and interquartile ranges. No changes were observed.
Blood pressure and heart rate

Mean intra-arterial pressure (MAP) did not change during either placebo or BNP infusion (p=0.475 and p=0.650, respectively; Figure 3.2). Whereas heart rate did not change during placebo infusion (p=0.505), it increased significantly from 67 beats per minute at baseline to 72 beats per minute at the highest dose of BNP infusion (p=0.011; Figure 3.2). The difference in heart rate responses between the placebo and the BNP group was, however, not statistically significant (0.05<p<0.10).

![Graph of heart rate and mean intra-arterial blood pressure](image)

Figure 3.2 Heart rate (HR, panel A) and mean intra-arterial blood pressure (MAP, panel B) at baseline and after infusion of placebo (open circles) or BNP (closed circles). Data are presented as median and interquartile ranges. * p<0.02, BNP infusion versus baseline.

Discussion

The present study shows that intrarenal BNP infusion in supine hypertensive patients does not induce significant changes in renal blood flow, despite increases in circulating levels of cGMP. In addition, we did not find BNP-related changes in the secretion of active renin and in creatinine extraction.

At baseline, median BNP levels of our hypertensive patients were almost 3-times higher than those we measured before in the forearm of healthy subjects. This difference corroborates previous studies that demonstrated elevated plasma BNP concentrations in hypertension, and
which may be related to left ventricular hypertrophy, and/or diastolic dysfunction.\textsuperscript{16,17}

Intra-arterial infusion of BNP caused an approximately 7-fold increase in renal venous BNP levels. Although this increase is to be expected, we also observed a comparable rise in arterial plasma BNP levels, indicating overflow of BNP into the systemic circulation. The slight increase in heart rate at the highest dose of BNP is compatible with this notion and, in the absence of changes in mean arterial blood pressure may point towards mild baroreceptor activation. The elevated BNP levels at the end of the infusion period approached but mostly exceeded the venous levels reached in systemic infusion studies.\textsuperscript{1,3-8} However, in this respect a simple comparison is not justified because the BNP levels reported here were obtained at the end of a dose-response study. Thus, in our experiment, the systemic vasculature must have been exposed to lower concentrations of BNP during the greater part of the study. Despite higher circulating BNP, the gradient of BNP across the kidney did not change. In fact, we found no evidence for renal extraction of BNP, neither at baseline nor during BNP infusion. This casts doubt on the supposition of a direct renal effect of BNP.

In a previous study, we demonstrated that intra-arterial BNP infusion into the human forearm induces a dose-dependent vasodilatation, via an increase in cGMP and c-type natriuretic peptide (CNP) levels.\textsuperscript{15} The second messenger cGMP is thought to be generated by activation of the natriuretic peptide receptor A (NPR-A) and/or via nitric oxide (NO) production. The present study shows that BNP infusion, indeed, induced cGMP release in our patients. Both renal venous and arterial cGMP plasma levels rose significantly after BNP infusion, but there was no change in the net renal cGMP gradient, with even a tendency for uptake rather than release. Taken together, these observations suggest that BNP by itself is not able to trigger cGMP release intrarenally and this may explain why we failed to observe renal vasodilatation. In addition, it suggests that cGMP was produced somewhere else in the cardiovascular system. Other variables to assess the effect of BNP, i.e. creatinine extraction (as marker for filtrating capacity) and renin secretion, also showed no differences after BNP infusion.

Another explanation for the lack of variation in MRBF may be that BNP, except for inducing vasodilatation, simultaneously stimulates a vasoconstrictor mechanism within the kidney and that any locally produced cGMP is excreted into the urine. Indeed, it has been demonstrated before that a close functional relationship exists between BNP and intrarenal endothelin-1 (ET-1) production.\textsuperscript{18} Apart from being a potent vasoconstrictor, ET-1 exhibits intrarenal natriuretic activity. In the present study we did not measure urine indices, but in literature systemic infusion of BNP is accompanied by an increased urinary excretion of ET-1, cGMP and sodium, without changes in plasma ET-1, plasma sodium, and plasma creatinine.\textsuperscript{16} Our previous finding that BNP is a mild dilator
Chapter 3

compared to equimolar doses of ANP supports the concept of a balance between vasodilating and vasoconstrictor forces. An additional argument may be that in in-vitro experiments, we were unable to demonstrate consistently BNP-induced vasodilatation in human and rat tissue. In phenylephrine-preconstricted human omental and pericardial resistance arteries, and rat mesenteric, renal, saphenous, and uterine arteries, in only 4 out of 14 experiments 10 nM BNP (human BNP-32 and rat BNP-45) caused a relaxation. On the contrary, 10 μM acetylcholine induced dilatation in all experiments (unpublished observations).

Limitations

One could argue that the Xenon-washout technique is not sensitive enough to detect increases in renal blood flow. However, in earlier studies we showed that both acetylcholine and adenosine induce renal vasodilatation, and that this was adequately detected with our method. So, if there was any (net) effect of BNP, it must, at least, have been very small. Another limitation of the present study is that, for ethical reasons, we could not study healthy subjects. It is well known that in diseases such as hypertension, several mechanisms may be altered and dysfunctional. Therefore, it is possible that BNP produces greater renal vasodilatation in normals and that our patients merely exhibited decreased sensitivity for this peptide. In addition, we cannot exclude the possibility that BNP-induced changes may be more pronounced in the upright position. Furthermore, creatinine extraction may not be the best marker for evaluating changes in GFR. Finally, we want to stress that so far our findings are only applicable to those hypertensive patients who fulfilled our selection criteria, i.e. the ones with treatment-resistant hypertension and/or target organ damage.

Perspectives

Further investigations are needed to determine BNP binding sites in the kidney, and the role of urinary cGMP excretion. In addition, more research is needed to investigate the role of ET-1 as a mediator of BNP-induced renal effects. Finally, the hypothesis needs to be tested that renal effects of BNP can only be elicited in the face of systemic hemodynamic changes.
References


Chapter 4

Selective endothelin-1 B receptor blockade inhibits basal, but not BNP-induced natriuresis

K van der Zander, DJ Webb, NR Johnston, JGR De Mey, AJHM Houben, PW de Leeuw
Abstract

Brain natriuretic peptide (BNP) and endothelin (ET-1) both exhibit natriuretic activity within the human kidney. Furthermore, they both act partly through activation of the endothelium NO-pathway. Since endothelin-1 may cause vasodilatation and natriuresis via stimulation of the ET-1 B receptor, the aim of the present study was to investigate in healthy subjects whether renal ET-B receptors participate in the renal actions of BNP.

In this placebo-controlled, cross-over study we infused BNP (4 pmol/kg/min) or placebo (i.v.) for 1 hour, with or without co-infusion of the endothelin B receptor antagonist BQ-788 (50 nM/min) for 15 min on 4 separate days, in 5 healthy subjects (mean age 50±5yr). Cardiac output was studied before and after infusion, using echocardiography.

Furthermore, during infusion we measured effective renal plasma flow (ERPF), and glomerular filtration rate (GFR) using PAH/inulin clearance. Blood pressure and heart rate (HR) were monitored as well. Urine and plasma samples were taken every hour to measure diuresis, natriuresis, cGMP and ET-1 levels.

BQ-788 decreased natriuresis, while BNP increased natriuresis, diuresis, GFR, FF and filtered load, without changing ERPF and filtration fraction. Neither BQ-788 or BNP altered cardiac output, blood pressure and heart rate. Combination of BNP and BQ-788 infusion showed no differences in hemodynamics and natriuresis as compared to BNP infusion alone.

The present study shows that selective ET-B receptor antagonism by itself decreases renal sodium excretion, but that it has no effect on the BNP-induced natriuresis and glomerular filtration rate.
Introduction

Brain natriuretic peptide (BNP), one of the family of natriuretic peptides, stimulates natriuresis and diuresis, but the renal mechanisms that are involved remain unclear. Increases in glomerular filtration rate (GFR) and renal plasma flow (RPF) could represent the intrarenal forces, which explain the enhanced natriuresis occurring during systemic BNP infusion. However, La Villa et al. found a natriuretic effect of BNP in the absence of changes in GFR and RPF in healthy subjects.\(^1\) Furthermore, we observed in hypertensive patients that intrarenal BNP infusion does not induce significant changes in renal blood flow or creatinine extraction (as a marker of glomerular filtration).\(^2\) This suggests that BNP primarily acts at the tubular level in the kidney. On the other hand, it may be that BNP, except for inducing renal vasodilatation, simultaneously stimulates a vasoconstrictor mechanism with the net effect that RPF remains constant. Indeed, it has demonstrated before that a close relationship exists between BNP and intrarenal endothelin-1 (ET-1) production.\(^3\) Apart from being a potent vasoconstrictor, ET-1 exhibits intrarenal natriuretic activity independently of changes in filtered load.\(^4\) The role of ET-1 in the kidney may even be dissociated from circulating ET-1. In fact, De Feo et al. demonstrated that systemic infusion of BNP is accompanied by increased urinary excretion of ET-1, cGMP and sodium, without changes in plasma ET-1.\(^5\)

ET-1 acts in an autocrine and paracrine manner on two subtypes of ET receptors, termed ET-A and ET-B. These receptors are located on vascular smooth muscle cells and binding of ET-1 to these sites results in sustained vasoconstriction. However, ET-B receptors are also present on endothelial cells where their activation leads to production of NO and vasodilator prostanooids, and subsequent vasodilatation. In a previous study, we demonstrated that BNP acts partly via the production of NO.\(^5\) Thus, it may be that the close relationship between BNP and ET-1 production in the kidney means that ET-1 mediates (part of) the effects of BNP via the ET-B receptor. If this were the case, this would introduce an interesting novel interaction for therapeutical interventions. Indeed, currently a number of drugs are under investigation, which either inhibit the enzyme that degrades natriuretic peptides (neutral endopeptidase inhibitors) or block endothelin receptors. Both types of agents are evaluated for their potential in the treatment of cardiovascular disease. Concurrent administration of a neutral endopeptidase inhibitor and a selective ET-A receptor antagonist would greatly enhance BNP’s effect on the kidney if ET-B receptors were to mediate these effects. The aim of the present study, therefore, was to test this hypothesis by investigating whether renal ET-B receptors are involved in the renal actions of BNP. To
this end, we studied the cardiac and renal effects of BNP with and without co-infusion of the ET-B receptor antagonist BQ-788 in a group of healthy subjects. Because the pathophysiological role of BNP becomes particularly evident in older patients we selected for this study only individuals above age 50 years.

Methods

Subjects

Experiments were performed in 5 healthy volunteers. During the week prior to the measurements, all subjects adhered to a 175 mmol Na+-containing diet so as to minimize variations in results due to differences in salt intake. Compliance with the diet was checked by measuring sodium and creatinine output in 24-hour urine collections obtained during the last 24 hours before the experimental day. None of the subjects used any medication (including non-steroidal anti-inflammatory drugs) during the two weeks prior to the measurements. In addition, they had to refrain from smoking and drinking caffeine or alcohol containing beverages for at least 12 hours before the experiments, which started at 8:30 a.m. after an overnight fast. The Medical Ethics Committee of the Maastricht University Hospital approved the study, and all participants gave written informed consent. The investigations conformed to the principles outlined in the Declaration of Helsinki.

Experimental design

The study was designed as a four way crossover trial. All volunteers were studied on four separate occasions (at least two days apart), during which they received in random order (double blind) an i.v. infusion of either vehicle (glucose 5%), BNP, a combination of placebo with BQ-788 or BNP combined with BQ-788. Experiments were performed in a quiet, temperature-controlled room. Precautions were taken to minimize external disturbances. Subjects remained supine throughout the experiments. A 20-gauge catheter was inserted into the antecubital vein of both arms. One was connected to a 3-way tap for infusion of placebo, BNP, BQ-788 (both from Clinifarma, Ethitorma Nederland BV, The Netherlands) and para-amino hippurate (PAH)/inulin (for measuring renal hemodynamics), while the other was used for blood sampling. To ensure adequate diuresis, subjects consumed 200 mL of water every hour until the last blood samples had been drawn. At \( t=0 \) min the PAH/inulin infusion was started and an echocardiogram was taken. At \( t=120 \) min the intravenous infusion of either placebo (glucose 5%), BNP (4 pmol/kg/min), a combination of placebo with BQ-788 (50nmol/min) or BNP combined with BQ-788 was started. Based on our previous
ETB antagonism and BNP in human kidney

studies, these relatively low doses of BNP and BQ-788 were suspected to influence renal, but not systemic hemodynamics. Infusion of BQ-788 stopped at t=135 min. At t=180 min (i.e. after one hour of placebo or BNP infusion), a second echocardiogram was performed. Blood pressure and heart rate (HR) were measured before and after the measurements in sitting position, and at 10-minute intervals during the infusion in supine position. Blood samples were drawn at t=0, t=120, and t=180 min for PAH and inulin and at t=120 and t=180 min for determination of hematocrit, cGMP and ET-1. Urine samples for measurement of sodium, cGMP and ET-1 were collected at t=60, t=120, and t=180 min (immediately after blood sampling).

Measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR were measured by an automatic oscillometric device [Dinamap Vital Signs Monitor 1846, Critikon]. CO was measured by echocardiography and total peripheral resistance (TPR) was calculated as \( \frac{MAP}{CO} \times 80 \) and expressed in dyne \( \cdot \) s/cm\(^2\).

Renal hemodynamics, i.e. effective renal plasma flow (ERPF) and glomerular filtration rate (GFR), were measured as the clearance of PAH (MSD, West Point, PA, USA) and inulin (Innotest, Laevonsan Gesellschaft, Linz, Austria) respectively, during continuous infusion of these substances. Both GFR and ERPF were corrected for body surface area and expressed as mL/(min \cdot 1.73 m\(^2\)). Effective renal blood flow (ERBF) was calculated by the following formula: \( \text{ERBF} = \frac{\text{ERPF}}{1-\text{hematocrit}} \). Filtration fraction (FF) was calculated as GFR/ERPF. Renal vascular resistance (RVR) was calculated as \( \frac{\text{MAP}}{\text{ERBF}} \times 80 \times 000 \) and expressed in dyne \( \cdot \) s/cm\(^5\). Renal fraction (RF) was calculated as \( \frac{\text{ERBF} \times \text{CO}}{100\%} \). Filtered load (FL) is calculated as GFR \times 60 \times [Na\(^+\)]\text{plasma} and expressed in mmol/hr. Fractional tubular reabsorption (FTR) is calculated as \( \frac{(\text{FL-}\text{U}_{\text{NaV}})}{\text{FL}} \times 100\% \), where \( \text{U}_{\text{NaV}} \) stands for the amount of Na\(^+\) excreted in the urine, and expressed also in mmol/hr.

Assay methods

PAH and inulin levels were measured by means of a spectrophotometer. cGMP levels were measured with a competitive protein-binding RIA (IBL Hamburg RE 29071). In our hands, the intra- and interassay variability of all assays was <10%. ET-1 was determined by standard RIA (Peninsula Laboratories Europe).

Statistics

As their distribution was not normal, data are presented as medians with interquartile ranges (IQR). The primary outcome variable in this study was the difference in natriuretic response during combined BNP/BQ-788
infusion relative to that during BNP alone. Secondary analyses comprised the effects of the interventions on renal hemodynamics. For each intervention, we calculated the percent change relative to pre-infusion values. Friedman's test (non-parametric two-way ANOVA) was used for analysis of multiple related samples (between visits) and Wilcoxon paired sign test for paired analysis (both within one visit and between visits). P values below 0.05 denote statistical significance. In case a Friedman's test demonstrated statistical significance, post hoc analyses were performed by a Wilcoxon paired sign test. Based on previous experiments we calculated that this study is able to demonstrate a 10% difference in any of the test variables with a power of 80% in 5 experimental subjects.

Results

Baseline clinical characteristics of the study participants are summarised in Table 4.1.

Table 4.1 Characteristics of the five study participants (median and IQR).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (male/female)</td>
<td>5 (3/2)</td>
</tr>
<tr>
<td>Age, year</td>
<td>60 (58-61)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.9 (25.8-27.4)</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>121 (120-126)</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>81 (79-84)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>69 (59-64)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mmol/24h</td>
<td>168 (115-234)</td>
</tr>
</tbody>
</table>

(blood pressure is the mean of 3 measurements by sphygmomanometer in sitting position)
BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HR, Heart Rate.

Plasma and urinary levels of cGMP and endothelin-1

At baseline, cGMP plasma levels did not differ between visits (p=0.564; Table 4.2). Both infusion of BNP and the combination of BNP and BQ-788 significantly increased the cGMP plasma level to 14.8 (13.5-19.8) pmol/mL and 22.5 (21.5-29.5) pmol/mL respectively (p<0.05), while placebo infusion with and without BQ-788 had no significant effect on cGMP plasma levels. The percent rise in plasma cGMP was significantly greater with infusion of BNP with or without co-infusion of BQ-788 (296 (168-418) % and 113 (92-370) % respectively) as compared to placebo.
with (35 [27.55] %) or without (29 [11.47] %) co-infusion of BQ-788 (p<0.05).

Baseline plasma ET-1 levels did not differ between visits (p=0.098; Table 4.2). Both infusion of placebo and BNP significantly increased ET-1 levels to 3.4 (3.0-3.9) pg/mL and 2.8 (2.7-4.1) pg/mL respectively (p<0.05), while infusion of placebo or BNP co-infused with BQ-788 had no significant effect on ET-1 plasma levels.

Whereas no effects, whatsoever, were seen with respect to urinary ET-1 excretion, changes in urinary excretion of cGMP followed the pattern of plasma levels.

Table 4.2 Baseline data of all variables (supine) before infusion of placebo, placebo combined with BQ-788, BNP, or BNP combined with BQ-788.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>Placebo/BQ-788</th>
<th>BNP</th>
<th>BNP/BQ-788</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cGMP, pmol/mL</td>
<td>5.2</td>
<td>5.7</td>
<td>6.0</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>(4.4-7.9)</td>
<td>(5.2-10.2)</td>
<td>(4.2-7.6)</td>
<td>(4.7-10.1)</td>
</tr>
<tr>
<td>Plasma ET-1, pg/mL</td>
<td>2.7</td>
<td>2.9</td>
<td>1.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>(2.5-3.0)</td>
<td>(2.5-3.1)</td>
<td>(0.6-2.7)</td>
<td>(2.3-4.5)</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>97</td>
<td>91</td>
<td>97</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>(95-103)</td>
<td>(87-97)</td>
<td>(92-102)</td>
<td>(88-100)</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>52</td>
<td>56</td>
<td>59</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>(51-59)</td>
<td>(48-58)</td>
<td>(55-65)</td>
<td>(48-58)</td>
</tr>
<tr>
<td>SV, mL</td>
<td>86</td>
<td>83</td>
<td>82</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>(84-109)</td>
<td>(74-96)</td>
<td>(70-99)</td>
<td>(72-96)</td>
</tr>
<tr>
<td>CO, L</td>
<td>5.4</td>
<td>4.7</td>
<td>4.9</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>(4.2-6.8)</td>
<td>(3.6-6.1)</td>
<td>(3.7-6.2)</td>
<td>(4.0-5.9)</td>
</tr>
<tr>
<td>TPR, dyne*s/cm²</td>
<td>1.535</td>
<td>1.432</td>
<td>1.759</td>
<td>1.547</td>
</tr>
<tr>
<td></td>
<td>(895-1 408)</td>
<td>(1 238-2 050)</td>
<td>(1 216-2 313)</td>
<td>(1 308-1 950)</td>
</tr>
<tr>
<td>ERPF, ml/min*1.73m²</td>
<td>458</td>
<td>434</td>
<td>432</td>
<td>423</td>
</tr>
<tr>
<td></td>
<td>(333-537)</td>
<td>(367-474)</td>
<td>(414-483)</td>
<td>(335-477)</td>
</tr>
<tr>
<td>ERBF, ml/min*1.73m²</td>
<td>755</td>
<td>750</td>
<td>751</td>
<td>693</td>
</tr>
<tr>
<td></td>
<td>(575-959)</td>
<td>(609-871)</td>
<td>(696-903)</td>
<td>(566-884)</td>
</tr>
<tr>
<td>GFR, ml/min*1.73m²</td>
<td>106</td>
<td>86</td>
<td>109</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>(94-132)</td>
<td>(82-102)</td>
<td>(95-123)</td>
<td>(79-101)</td>
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<tr>
<td>FF, %</td>
<td>25</td>
<td>21</td>
<td>23</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>(22-30)</td>
<td>(19-25)</td>
<td>(22-28)</td>
<td>(20-26)</td>
</tr>
<tr>
<td>RVR, dyne*s/cm²</td>
<td>9.314</td>
<td>10.649</td>
<td>7.886</td>
<td>11.769</td>
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<tr>
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<td>(4.933-9 037)</td>
<td>(6 428-11 465)</td>
<td>(6 701-10 969)</td>
<td>(6 443-12 938)</td>
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<tr>
<td>RF, %</td>
<td>17</td>
<td>17</td>
<td>21</td>
<td>16</td>
</tr>
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<td></td>
<td>(11-22)</td>
<td>(14-21)</td>
<td>(14-23)</td>
<td>(14-21)</td>
</tr>
<tr>
<td>U₁RUV, mmol/hr</td>
<td>8.1</td>
<td>10.3</td>
<td>6.6</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>(7.8-12.3)</td>
<td>(7.1-16.7)</td>
<td>(5.9-10.0)</td>
<td>(4.8-21.4)</td>
</tr>
<tr>
<td>V, ml/hr</td>
<td>70</td>
<td>160</td>
<td>70</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>(58-225)</td>
<td>(75-420)</td>
<td>(65-223)</td>
<td>(195-438)</td>
</tr>
<tr>
<td>FTR_ex, %</td>
<td>99.1</td>
<td>99.0</td>
<td>99.4</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>(99.0-99.1)</td>
<td>(98.8-99.0)</td>
<td>(98.9-99.5)</td>
<td>(97.7-99.1)</td>
</tr>
</tbody>
</table>

Data are expressed as medians and IQR. *p<0.05 versus BNP.
Systemic hemodynamic effects

At baseline, no differences in blood pressure (MAP), heart rate (HR), stroke volume (SV), cardiac output (CO) and total peripheral resistance (TPR) were observed between the experiments (Table 4.2). MAP and HR were not significantly altered by infusion of placebo, the combination of placebo/BQ-788, BNP, or the combination of BNP/BQ-788 (Figure 4.1). Furthermore, no differences were observed between interventions. CO did not significantly change either during any of the infusions and there were also no differences between visits. Finally, changes in TPR did not differ between the four visits.

![Graph A](image1)

**Figure 4.1.** Supine heart rate (HR, panel A) and mean arterial blood pressure (MAP, panel B) at baseline and every 10 minutes during 1 hour infusion of placebo or BNP with or without co-infusion of BQ-788 for 15 minutes. Data are presented as median and interquartile ranges.
Renal effects

Baseline GFR was not the same for the four visits (p=0.022). On the
days that BQ-788 was administered baseline GFR was lower than before
infusion of BNP alone (Table 4.2), but otherwise baseline renal
hemodynamic variables did not differ between visits. GFR significantly
increased during infusion of BNP, both when given alone or in the
presence of BQ-788 (Figure 4.2). Except for a rise in FF after BNP
infusion alone, no changes in ERPF and FF were observed during any
infusion (Figure 4.2). No differences or changes in RVR and RF were
observed between visits.

![Graphs A, B, C, and D showing changes in renal variables over time.]

Figure 4.2. Renal hemodynamic responses of renal plasma flow (ERPF, panel A),
glomerular filtration rate (GFR, Panel B), filtration fraction (FF, Panel C), and
sodium excretion (Na+, panel D) to intravenous infusion of placebo or BNP
infusion with or without co-infusion of BQ-788. Data are expressed as
percentage change and presented as median and IQR. * p<0.05 versus
baseline. " p<0.05 between interventions.

At baseline, \( U_{Na} \) and urinary volume were comparable for all visits
(Table 4.2). BNP with or without co-infusion of BQ-788 induced a
significant natriuresis. Urinary sodium excretion in the one-hour urine
collection remained unchanged during the placebo experiment, but fell
significantly during placebo/BQ-788 infusion to 7.9 (5.6-13.3) mmol
(p=0.043; Figure 4.2) Sodium excretion increased to 29.0 (16.8-45.2) mmol and to 26.6 (19.7-34.6) mmol respectively during BNP with and without BQ-788 (Figure 4.2). Natriuretic responses did not differ when BNP alone was compared to BNP/BQ-788 (p=0.345; Figure 4.2). The urinary volume of the one-hour urine collection significantly rose to 460 (378-743) mL after BNP infusion, while it did not increase significantly during the other infusions in spite of the water suppletion during the renal clearance study protocol. However, percent changes in diuresis did not differ between BNP alone and during combination of BNP/BQ-788. FL Na increased significantly from 1048 (792-1313) mmol/hr to 1116 (829-1412) mmol/hr during BNP and from 861 (664-1037) mmol/hr to 905 (706-1287) mmol/hr during co-infusion of BNP and BQ-788, while placebo with or without BQ-788 infusion did not influence FL Na. Furthermore, BNP significantly decreased FTR Na (without BQ-788 to 97.4 (97.1-98.2) % and with BQ-788 to 96.8 (96.1-97.9) %) as compared to placebo (p=0.019), there being no differences between the two experiments with or without co-infusion of BQ-788. No difference in FTR Na was observed between the combination of placebo/BQ-788 and infusion of placebo alone or between BNP/BQ-788 and BNP infusion alone.

Discussion

The present study was designed to test the hypothesis that the renal effects of BNP (enhanced GFR and natriuresis) are mediated by ET-B receptors. However, while our data indicate that selective ET-B receptor antagonism by itself decreases renal sodium excretion, we found no evidence for an effect of ET-B receptors on BNP-induced natriuresis or diuresis. Moreover, the rise in glomerular filtration rate as observed during administration of BNP is not influenced by ET-B receptor blockade.

The rise in plasma ET-1 levels which we observed after BNP infusion probably reflects a spontaneous phenomenon related to our study design or to diurnal changes, since this increase is also observed after placebo infusion. Selective ET-B receptor blockade appeared to prevent this rise in plasma ET-1, which is surprising because one would expect an even greater rise because the peptide is normally cleared by the ET-B receptor. Although we cannot readily explain these observations, it is unlikely that changes in plasma ET-1 are relevant for the present results. Strachan et al. demonstrated substantial systemic vasoconstriction, associated with a reduction in HR and cardiac index, but no change in MAP, in response to administration of the selective ET-B receptor antagonist BQ-788 in healthy men. These effects were most prominent with the highest dose of BQ-788 (300 nmol/min), but were not clearly
seen at lower doses. In the present study we employed a low dose of the antagonist and did not observe any significant changes in systemic hemodynamics. Therefore, it is unlikely that renal effects of BQ-788 in this study are related to systemic vasoconstriction.

Experiments in anaesthetised rats have shown that low doses of intravenous ET-1 cause natriuresis due to reduced sodium transport in the proximal and distal nephron segments and that higher doses result in sodium retention due to glomerular vasoconstriction. Data obtained with ET-A and ET-B specific antagonists in anaesthetized dogs indicate that the ET-A receptor predominantly accounts for renal vasoconstriction, while the ET-B receptor is largely responsible for diuresis and natriuresis. The results of our human study are in line with these animal experiments, since low-dose ET-B blockade decreased urinary sodium excretion, without changing ERPF, GFR, and FF. We could not demonstrate a BQ-788 induced increase in fractional tubular sodium reabsorption in man, but for this to become apparent, one probably needs larger groups of subjects.

Previous investigations have demonstrated that BNP is able to induce a dose-dependent vasodilatation through activation of the natriuretic peptide receptor A (NPR-A), with an associated increase in cGMP, as well as by production of nitric oxide (NO). In line with our previous work, the present data confirmed that BNP infusion enhanced the release of plasma and urinary cGMP, urinary sodium excretion, and urine volume along with a significant increase in GFR, FF and filtered load of sodium. Furthermore, BNP infusion did not change systemic hemodynamics either. Therefore, it is fair to conclude that the dose of BNP, which we used, was able to activate its receptors and enhance the release of its second messenger cGMP, but low enough to keep systemic hemodynamics unaffected.

BNP could increase GFR in three possible ways, namely by pre-glomerular vasodilatation, by post-glomerular vasoconstriction, and/or by changing filtration surface area. The first possibility, pre-glomerular vasodilatation, is not very likely to have occurred since we did not notice any changes in ERPF. Of course, we cannot exclude that BNP augments filtration surface area, but presently there are no good tools to investigate this in an unbiased way. For the time being, therefore, we consider post-glomerular vasoconstriction the most likely mechanism of the BNP-induced rise in GFR.

None of the effects of BNP were modified by concurrent administration of the ET-B receptor antagonist. Nevertheless, an obvious limitation in this study is that we could not assess the degree of ET-B receptor blockade. Indeed, infusion of ET-1, to demonstrate a shift in dose-response curve, would not only stimulate ET-B but also unoccupied ET-A receptors, making unambiguous conclusions impossible. However, the fact that ET-B receptor blockade was associated with anti-natriuresis suggests that we did achieve adequate blockade.
Irrespective of the mechanisms, the intrarenal hemodynamic and natriuretic effects of BNP were not affected by the anti-natriuretic action of BQ-788. It is possible that the renal effect of BNP overruled ET-B receptor antagonism, but this is unlikely since we used a low dose of BNP. Therefore, we conclude that ET-B receptors are not involved in the BNP-induced stimulation of GFR.
References


Chapter 5

Effects of Brain Natriuretic Peptide on forearm vasculature: comparison with Atrial Natriuretic Peptide

K van der Zander, AJHM Houben, AA Kroon, PW de Leeuw
Abstract

The aim of the present study was to determine the vasoactive effects of brain natriuretic peptide (BNP) as compared to those of atrial natriuretic peptide (ANP) in normal man. Ten healthy male subjects (median age 21 [20-23] year) were studied twice. In the first study equimolar doses (1, 3, and 10 pmol/dl/min) of both BNP and ANP (in random order and double blind) were infused into the brachial artery of the non-dominant arm with a 1-hour washout period in between. In the second study two BNP (n=5) or ANP (n=5) dose-response curves were performed in order to assess the repeatability of the BNP/ANP infusions. To this end, BNP and ANP were infused in the same equimolar doses as in the first protocol. Forearm blood flow (FBF) was determined by venous occlusion plethysmography before and during infusions.

BNP increased the FBF ratio (infused/contralateral arm) by 6%, 17%, and 48%, respectively (p<0.05), while ANP increased the FBF ratio by 4%, 58%, and 133% (p<0.001). The slopes of the BNP dose-response curves differed significantly from those of the ANP curves (18.1 versus 43.2; p=0.022). No differences were observed between the repeated dose-response curves of either BNP or ANP.

The present data demonstrate that BNP induces a dose-dependent vasodilatation in man. On a molar basis, however, this vasodilatation is significantly less than the vasodilatation induced by ANP. These differences may be related to differences in natriuretic-peptide-receptor affinity. Furthermore, our data show that the vasoactive effects of both BNP and ANP are repeatable in time.
Introduction

Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP), two members of the family of natriuretic peptides, stimulate sodium excretion by the kidney. BNP is mainly produced in the ventricles of the heart, while ANP is secreted from the atria. However, the mechanisms controlling the release of ANP and BNP may be different for the two peptides. This is corroborated by the finding that intravascular saline loading acutely increases the plasma concentrations of ANP without any effect on plasma BNP concentrations. On the other hand, increased BNP levels are associated with left ventricular hypertrophy (LVH), and reduced cardiac output. Hence, BNP may be an marker for LVH or left ventricular dysfunction. It may be, therefore, that BNP levels more reflect long-term intravascular volume status rather than momentary volume changes.

ANP and BNP not only stimulate natriuresis and diuresis, but also cause vascular relaxation. While the renal effects of these two peptides when given in equimolar doses seem to be comparable, little is known about their relative potencies at the level of the vascular wall. In fact, the hemodynamic effects of BNP are somewhat contradicting. For instance, in chronic heart failure systemic infusion of BNP results in a fall in blood pressure, systemic vascular resistance, and pulmonary capillary wedge pressure. On the other hand, studies in both hypertensive patients and healthy subjects reveal no effects of BNP on blood pressure, cardiac output or systemic vascular resistance. This difference in results could be related, however, to the BNP levels which were reached.

All previously described effects of BNP and ANP have been derived mainly from animal and human experiments using systemic infusions, although local vascular effects of ANP have been reported as well, the direct vascular effects of BNP have been studied in less detail. In vitro studies suggest that BNP exerts its biological effects through the same pathway as ANP does, i.e. the natriuretic peptide receptor A (NPR-A). However, the affinity of BNP for this receptor is less than that of ANP. Because of this difference in affinity, we suspected that ANP will cause a greater degree of vasodilatation than BNP when given in equimolar doses in man. The aim of the present study was, therefore, to compare the local vascular effects of BNP to those of ANP in healthy men. In addition, we wanted to determine the repeatability of the effects of BNP and ANP.
Chapter 5

Subjects and methods

Subjects

Experiments were performed in 10 healthy male volunteers, with a median age of 21 (interq. range 20-23) years. One week prior to the measurements, subjects followed an ad libitum salt diet, which resulted in a median 24h urinary sodium excretion of about 140 mmol. They were asked to refrain from smoking and caffeine or alcohol containing beverages for at least 12 hours before the experiments, which started at 8 a.m. after an overnight fast.

Furthermore, none of the subjects had used any medication (including non-steroidal anti-inflammatory drugs) in the two weeks prior to the measurements.

The study was approved by the Medical-Ethics Committee of the Maastricht University Hospital, and all participants gave written informed consent. The investigations conform with the principles outlined in the Declaration of Helsinki.11

Experimental design

All volunteers were studied twice with a 2-week interval. The order of the experiments was randomised. Subjects were studied in supine position in a quite, temperature-controlled room (mean temp. 24.1±0.2°C). A 20-gauge catheter was inserted into the brachial artery of the non-dominant arm (under local anaesthesia, 1% lidocaine): for infusion of drugs and monitoring of blood pressure. Forearm volume was measured by water displacement; drug infusion rates were normalised to 100 ml forearm tissue.

In the first study, forearm vascular reactivity to repeated dose-response curves of ANP (N=5) or BNP (N=5) was studied. In the second study, forearm vascular reactivity to infusion of equimolar doses of both ANP and BNP was studied. These infusions were performed in random order and in a double blind fashion. The two studies were performed according to a similar experimental design.

Equimolar peptide doses of 1, 3, and 10 pmol/100ml forearm/min were used in both studies, which was based on data obtained in pilot experiments as well as on data from literature.12 Each dose was infused for 5 minutes. Forearm blood flow (FBF) was determined simultaneously in both arms using ECG-triggered venous occlusion plethysmography (ID-Plethysmograph, University of Maastricht, The Netherlands), as described in detail previously.13 Blood pressure was measured intrarterially using a Hewlett Packard 78205C monitor. Heart rate was derived from the ECG.

Basal measurements of blood pressure, heart rate, and FBF were obtained 15 minutes after insertion of the arterial catheter. Following another 15 minutes the first dose-response curve was determined, with
FBF being recorded continuously from 2 minutes before until the end of infusion of BNP/ANP. The mean value of the last minute of each dose (0, 1, 3, and 10 pmol/dl/min) was used for the analyses. Before the next infusion of BNP/ANP, there was a 1-hour recovery period in order to allow forearm blood flow (FBF) to return to baseline values. All signals were stored on the hard disk of a personal computer by means of a custom-built data acquisition system.

Drugs

All solutions were freshly prepared in 5% glucose immediately before infusion. ANP and BNP were obtained from Clinalfa (Ethisfarma Nederland BV, The Netherlands).

Statistics and calculations

For each measurement of FBF the ratio between the infused arm and the contralateral arm was calculated. This ratio corrects for all systemic factors that affect the regulation of blood flow in both arms (e.g. changes in blood pressure, level of arousal, hormonal changes, etc.), and is stable during the day. Furthermore, it ensures that the direct effects of locally infused substances on forearm blood flow can be assessed. For each separate dose of drug the percentage change in FBF ratio (relative to pre-infusion values) was calculated. This calculated value is less influenced by possible changes in FBF.

Besides calculation of ANP/BNP dose-response curves for each individual, also individual concentration-response curves were calculated by correcting the dose for FBF at the time of infusion. The slopes of both the dose-response and the concentration-response curves were calculated individually by linear regression of the percentage change in FBF ratio during the three doses of ANP/BNP. These slopes summarise each individual response to the three doses in one number. Statistically, this calculation will avoid the problem of repeated measures between the three doses.

Since the distribution of the FBF data within the group was not normal, data are presented as median values with interquartile ranges. Friedman’s test (non-parametric two-way ANOVA) was used for analysis of multiple related samples (within one visit) and Wilcoxon paired sign test for paired analysis (between visits and within one visit). When appropriate, the Bonferroni correction was used for multiple comparisons. P values below 0.05 were considered statistically significant.

To examine the repeatability of the effects of both hormones, the Bland-Altman method was applied.
Chapter 5

Results

The baseline clinical characteristics of the ten male study participants are summarised in Table 5.1. Median urinary sodium output in the 24 hours prior to the first study was 139 (111-172) mmol, and prior to the second study 143 (91-196) mmol, indicating that salt intake of the subjects was not significantly different between the two experiments.

Table 5.1 Characteristics of the ten study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21 (20-23)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>79 (71-83)</td>
</tr>
<tr>
<td>Length, m</td>
<td>1.83 (1.80-1.85)</td>
</tr>
<tr>
<td>Body Mass Index, kg/m²</td>
<td>23.6 (21.3-24.4)</td>
</tr>
<tr>
<td>Heart Rate, beats per min</td>
<td>55 (50-57)</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>85 (77-90)</td>
</tr>
</tbody>
</table>

* Values are presented as median and interquartile range

Blood pressure and heart rate

MAP and HR did not change during any of the studies (Table 5.2), indicating that the local infusions of BNP or ANP had no systemic hemodynamic effects.

Table 5.2 Heart rate and mean arterial pressure of the subjects at the beginning and end of the two studies

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate (beats per min)</th>
<th>Mean Arterial Pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>Study 1</td>
<td>54 (51-57)</td>
<td>56 (50-60)</td>
</tr>
<tr>
<td>Study 2</td>
<td>55 (49-58)</td>
<td>53 (50-59)</td>
</tr>
</tbody>
</table>

* Blood pressure was measured intra-arterially. Values are presented as median and interquartile range.

Study 1: Repeatability of the effects of BNP/ANP

The median slopes of the two dose- or concentration-response curves, were comparable both for BNP and for ANP (p>0.05). Using the Bland-Altman approach, the mean difference in individual slopes of repeated dose-response curves for BNP is 5.5 (95 % confidence interval (C.I.) -22.6 to 33.7) and 0.8 (C.I. -6.5 to 8.2) for ANP. The mean difference in maximum percentage change in FBF of repeated infusions is 16.0 % (C.I. -77.0 to 109.0) for BNP and -6.9 % (C.I. -19.5-5.6) for ANP.

58
Study 2: Comparison of the effects of BNP and ANP on forearm blood flow

Since there was no evidence for a time-treatment interaction, the data from the randomised infusions were pooled for the analysis. The three doses of BNP increased FBF ratio by 6% (4-23), 17% (3-33), and 48% (7-87), respectively, relative to baseline (p<0.05; Figure 5.1). The percentage change in baseline FBF ratio for ANP was 4% (5-19), 58% (20-93), and 133% (54-173), respectively (p<0.001; Figure 5.1). In Table 5.3 the absolute flow (FBF) values and FBF ratio at baseline and after each dose of BNP/ANP are summarised.

![Graph showing change in FBF ratio (%) vs infused doses (pmol/dl/min)](image)

**Figure 5.1** Vasoreactivity of forearm (muscle) vasculature to local BNP and ANP infusions (i.a.) (expressed as percent change in FBF-ratio [infused/contralateral arm]). Data are presented as median and interquartile range. Both BNP and ANP caused significant dilatation (p<0.05). * P<0.05 BNP versus ANP.

Post hoc analysis of the three doses separately revealed that at the highest dose (10 pmol/dl/min) the effects of BNP and ANP were significantly different (p=0.022; Figure 5.1). The median slope of the regression line through the individual dose–response curves was 18.1 (4.7-28.3) for BNP and 43.2 (19.8-60.8) for ANP (p=0.022; Figure 5.2A). The median slope of the regression line through the concentration–response curves was 7.2 (0.3-16.8) for BNP and 25.5 (12.9-40.8) for ANP (p=0.017; Figure 5.2B).
Table 5.3 Absolute FBF values and FBF ratio after each infused dose^a^b^c^d

<table>
<thead>
<tr>
<th>Dose</th>
<th>FBF Infused arm (pmol/dl/min)</th>
<th>FBF Contralateral arm (ml/dl/min)</th>
<th>FBF-Ratio (=inf./cl) (ml/dl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP</td>
<td>baseline 2.7 [2.5-3.3]</td>
<td>2.6 [2.2-3.5]</td>
<td>1.15 [0.90-1.65]</td>
</tr>
<tr>
<td></td>
<td>1 3.0 [2.7-3.3]^b^d</td>
<td>2.8 [2.3-3.7]</td>
<td>1.29 [1.00-1.66]</td>
</tr>
<tr>
<td></td>
<td>3 3.2 [2.7-3.9]^b^d</td>
<td>2.9 [2.0-4.2]</td>
<td>1.43 [0.86-1.86]^b^d</td>
</tr>
<tr>
<td></td>
<td>10 4.2 [3.8-5.0]^b^d</td>
<td>2.9 [2.3-4.6]</td>
<td>1.46 [0.86-1.86]^b^d</td>
</tr>
<tr>
<td>ANP</td>
<td>baseline 2.8 [2.2-3.2]</td>
<td>2.7 [2.3-3.6]</td>
<td>0.86 [0.82-1.17]</td>
</tr>
<tr>
<td></td>
<td>1 3.0 [2.4-3.7]</td>
<td>3.1 [2.3-3.7]</td>
<td>1.08 [0.83-1.22]</td>
</tr>
<tr>
<td></td>
<td>3 5.0 [3.2-6.0]^e</td>
<td>3.5 [2.5-3.9]</td>
<td>1.49 [1.11-1.95]^f^</td>
</tr>
</tbody>
</table>

Figure 5.2 Slope of the regression line through the individual dose-response curves (A) and the individual concentration-response curves (B) during BNP and ANP infusions. Data are presented as median and interquartile range. * P<0.05 BNP versus ANP.

Discussion

The present data demonstrate that local BNP infusion in man induces a dose-dependent vasodilatation, which is significantly less than that induced by equimolar doses of ANP. Since the vasoactive effects of both...
BNP and ANP are repeatable in time, the difference between both peptides seems to be genuine and not due to some aspecific effect. Although we did not measure cardiac output, the design of the study and the use of FBF-ratio rather than absolute FBF values, make it extremely unlikely that the difference in effect between BNP and ANP can be explained by differential systemic effects of the two peptides. In addition, no changes in blood pressure and heart rate were apparent, which also argues against systemic effects of the agents.

Previous studies have shown that human BNP is able to relax human artery and vein tissue.\textsuperscript{16} In healthy human beings and patients with chronic heart failure Nakamura \textit{et al.}\textsuperscript{19}, using much higher doses than we did, also found a peripheral vasodilating effect of BNP.

At present, three different natriuretic peptide receptors (NPR) have been described: NPR-A, NPR-B, and NPR-C.\textsuperscript{9,23} The NPR-A and NPR-B are transmembrane guanylate cyclases, but the NPR-C is a short transmembrane protein which functions through intermediate G-proteins to inhibit adenylyl cyclase and stimulate the phosphoinositol pathway. The latter has been called the clearance receptor, but binding to this receptor results in biological activity as well.\textsuperscript{9,20,21} Both BNP and ANP are thought to act through the NPR-A. Activation of this receptor generates cGMP, which as second messenger activates Ca\textsuperscript{2+}-activated and ATP-sensitive K\textsuperscript{+}-channels, finally resulting in vasorelaxation.\textsuperscript{22,23}

The fact that BNP-induced vasodilatation is significantly less than that induced by equimolar doses of ANP could be explained by a difference in receptor-affinity. Cell culture experiments (human tissue cells) have shown that the affinity of BNP for NPR-A is 4-70 times less compared to ANP.\textsuperscript{9,10} Consequently, BNP is 10-fold less potent than ANP to stimulate cGMP production via the NPR-A.\textsuperscript{9}

There is, however, a body of evidence in the literature that does not support the concept of lesser affinity of the NPR-A for BNP. For instance, Nakamura \textit{et al.}\textsuperscript{19} found no difference in forearm vasorelaxation between BNP and ANP in heart failure patients, which they explained by a downregulation of the NPR-A in these patients. However, when both ANP and BNP act via the same receptor, such a downregulation would effect the BNP-induced dilatation in a similar proportional manner. Moreover, Procter \textit{et al.}\textsuperscript{18} showed that human BNP and ANP are equipotent in relaxing isolated preconstricted human arteries. Also in cultured endothelial cells BNP and ANP show a similar dissociation constant and maximal binding capacity for the NPR-A,\textsuperscript{24} and both peptides are equipotent in stimulation of endothelial cGMP production.\textsuperscript{25} BNP even induces a 20-fold greater release of CNP by endothelial cells than ANP.\textsuperscript{26} The latter effect was in case of ANP completely and in case of BNP partly cGMP-mediated. Finally, Moritoki \textit{et al.}\textsuperscript{27} demonstrated in young (4 weeks old) animals that ANP-induced vasodilatation is partly mediated via nitric. Hence, these findings suggest the possibility that BNP
and ANP act partly through different mechanisms, as a result of which the dilatory effect of BNP may be less than that of ANP. An alternative explanation may be that BNP, except for inducing vasodilation, also stimulates a vasoconstrictor mechanism. Although there are no data available to support such a hypothesis, it is well known that ANP can act as a vasoconstrictor. In previous studies we found that low doses of ANP into humans resulted in vasoconstriction of the microcirculation, most likely on the venular side. A comparable observation has been made in renal studies. ANP dilates preglomerular (afferent) arterioles and constricts postglomerular (efferent) arterioles, thus causing an increased hydraulic pressure in the glomerular capillaries. If BNP also has a dual action on the vasculature, the net effect of this peptide will depend on the balance between vasodilating and vasoconstrictor forces. Any difference in efficiency between BNP and ANP could thus depend on differences in this balance.

Plasma levels of BNP are lower than those of ANP in normal human subjects. In several disease states such as chronic heart failure (CHF) and hypertension, in particular when LVH is present, levels of both peptides are elevated. Moreover, BNP levels frequently surpass plasma levels of ANP in severe CHF. Thus, in view of our findings, it may be that BNP must increase to a greater extent than ANP in order to induce effective vasodilation, and maintain circulatory homeostasis.

In conclusion, our data demonstrate that both BNP and ANP induce vasodilatation of the forearm vasculature of healthy men. However, the effect of BNP is less potent in comparison with ANP. The difference in effect may be related to a difference in affinity for the natriuretic-peptide-receptor-A, or to a different balance in vasodilator and vasoconstrictor effects. Further studies are needed to elucidate the mechanisms of action of BNP.
References


Chapter 6

Nitric Oxide and potassium channels are involved in Brain Natriuretic Peptide induced vasodilatation in man

K van der Zander, AJHM Houben, AA Kroon, JGR De Mey, PABM Smits, PW de Leeuw
Abstract

Brain natriuretic peptide (BNP) causes vasodilatation but the mechanisms by which this is accomplished are not fully known. The aim of the present study was to determine whether besides \( \text{K}^+_{\text{Ca}^{2+}} \)-channels, nitric oxide (NO) is involved in BNP-induced vasodilatation. We studied ten healthy males twice, in random order, at a 2 weeks interval. Experiments always started with infusion of BNP (8-16-32-64 pmol/dl/min) into the brachial artery. On one day this infusion was followed by a second BNP infusion combined with the \( \text{K}^+_{\text{Ca}^{2+}} \)-channel-blocker Tetra-Ethyl-Ammonium (TEA, 0.1 mg/dl/min) and on the other day by BNP infusion combined with the NO-synthase inhibitor L-N^\text{N}-monomethyl arginine (L-NMMA 0.8 \mu mol/dl/min). The latter was then followed by a combined infusion of BNP, L-NMMA and TEA. All infusions were separated by a 1-h-washout period. Forearm blood flow (FBF) was determined by venous occlusion plethysmography.

Mean arterial pressure and heart rate did not change during any of the experiments. BNP alone induced a dose-dependent dilatation, which was similar on both days. TEA, L-NMMA, and their combination all reduced the BNP-induced dilatation (p<0.05). The combined infusion had a significantly greater effect than TEA alone (p=0.005). BNP infusions were associated with a significant increase in plasma cGMP and C-type natriuretic peptide (CNP) (p<0.05).

BNP induces arterial vasodilatation not only by opening \( \text{K}^+_{\text{Ca}^{2+}} \)-channels, but also via stimulation of NO production. In addition, BNP stimulates net CNP increase.
Introduction

A variety of data suggests that atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) constitute a dual natriuretic peptide system that responds differently to short- and long-term volume load.\textsuperscript{1,2} Patients with essential hypertension who develop left ventricular hypertrophy or frank heart failure usually have elevated plasma levels of BNP\textsuperscript{3,4}, but the significance of this finding is not well understood. Given the recent demonstration that intravenous administration of BNP improves hemodynamic function and clinical status in patients with decompensated congestive heart failure (CHF)\textsuperscript{5,6}, it is important, therefore, to further unravel the mechanisms by which this peptide acts. This would not only increase our knowledge about the pathophysiological significance of the hormone but potentially also offer new targets for pharmacotherapeutic modulation.

In vitro studies suggest that BNP exerts its vascular effects through the same pathway as ANP, i.e. the natriuretic peptide receptor A (NPR-A). This guanylate cyclase receptor is located both on endothelial and vascular smooth muscle cells.\textsuperscript{7,8} Activation of this receptor generates cGMP, which as second messenger activates Ca\textsuperscript{2+}-activated and ATP-sensitive potassium (K\textsuperscript{+})-channels, thus promoting vasorelaxation.\textsuperscript{9,10} Furthermore, both BNP and ANP can stimulate CNP production and secretion by endothelial cells.\textsuperscript{7} CNP reacts with its specific guanylate cyclase receptor on the vascular smooth muscle cell, also causing vasodilatation through hyperpolarisation.\textsuperscript{11}

Despite an apparent similar pathway of action for both peptides, we and others have shown that in healthy man BNP-induced vasodilatation is significantly less than that induced by equimolar doses of ANP.\textsuperscript{12,13} Such a discrepancy in effect could be related to a difference in affinity for the NPR-A\textsuperscript{14,15}, but not all data in the literature are compatible with this explanation. For instance, Nakamura et al.\textsuperscript{13} found that the difference in vasodilatation between BNP and ANP as observed in healthy controls was not present in patients with CHF. This has been explained by a downregulation of receptor (NPR-A) density in these patients.\textsuperscript{16} However, when both ANP and BNP would act through the same receptor such a downregulation should affect BNP-induced dilatation in a similar and proportional manner.

Based on experiments which showed that BNP-induced vasodilatation of pig coronary arteries could be blocked by nitric oxide (NO) synthase inhibition\textsuperscript{7}, and the finding of a close relationship between the basal production of NO in CHF patients and the severity of heart failure as reflected by plasma BNP levels\textsuperscript{18}, we hypothesised that BNP acts partly via the NPR-A, and partly via another (endothelium-dependent)
mechanism involving NO. It is, however, unclear whether this NO-involvement in BNP vasodilatation is NPR-A dependent or not. The aim of the present study was, therefore, to assess to what extent calcium-activated potassium channels (K_{Ca2+}-channels) and/or NO are involved in BNP-induced vasodilatation in man. Studies were carried out in the forearm skeletal muscle vascular bed of healthy subjects.

Methods

Subjects

Experiments were performed in 10 healthy male volunteers (median age 20 [range 18-24] years). In the week prior to the measurements, subjects ate a 150 mmol Na+-containing diet to minimize variations in results due to salt intake. In addition, they had to refrain from smoking and drinking caffeine or alcohol containing beverages for at least 12 hours before the experiments, which started at 8 a.m. after an overnight fast. None of the subjects used any medication (including non-steroidal anti-inflammatory drugs) during the two weeks prior to the measurements. The Medical Ethics Committee of the Maastricht University Hospital approved the study, and all participants gave written informed consent. The investigations conformed to the principles outlined in the Declaration of Helsinki.

Experimental design

All volunteers were studied twice with a 2-week interval and in randomised order. During the experiments, subjects remained supine in a quiet, temperature-controlled room (mean temperature 24.0±0.2°C). Two catheters were inserted into the non-dominant arm: one 20-gauge catheter into the brachial artery (local anaesthesia, 1% lidocaine) for infusion of drugs and monitoring of blood pressure, and one into the antecubital vein for blood sampling. Forearm volume was measured by water displacement and drug infusion rates were normalised to 100 ml forearm tissue. Both visits started with an infusion of BNP into the brachial artery followed by a one-hour recovery period to allow forearm blood flow to return to baseline levels (Figure 6.1). On the first experimental day, we then infused BNP again, but this time in combination with the K_{Ca2+}-channel-blocker Tetra-Ethyl-Ammonium chloride (TEA, 0.1 mg/dl/min). On the other experimental day, the second infusion consisted of BNP in combination with the NO-synthase inhibitor L-N^6-monomethyl arginine (L-NMMA) in a dose of 0.8 μmol/dl/min. This was then followed by an infusion of BNP in combination with L-NMMA and TEA. All experiments were performed in a similar way. Before infusion and at the end of the highest dose of BNP,
we drew venous blood samples in tubes containing an EDTA/trasylol mixture for analysis of BNP, C-type natriuretic peptide (CNP), and cGMP levels. Previously published data as well as pilot experiments indicated that the doses of TEA and L-NMMA employed in this study are the maximum ones that inhibit K_rk_A channels and NO-synthase (NOS) respectively without producing systemic hemodynamic effects.

Figure 6.1 Vasoreactivity of forearm (muscle) vasculature to local intra-arterial infusions of BNP with and without inhibitor(s), expressed as percent change in FBF ratio (infused/contralateral arm). Data are presented as medians. In all conditions BNP caused significant vasodilation (p<0.05). For abbreviations: see figure 1. BNP1 and BNP2: first and second BNP infusion respectively. * p<0.05 versus BNP infusion alone. † p=0.005 versus BNP+TEA.

Based on data obtained in a pilot study as well as on data from the literature, we infused BNP at doses of 8, 16, 32, and 64 pmol/100ml forearm/min, each dose for 3 minutes. Forearm blood flow (FBF) was determined simultaneously in both arms using ECG-triggered venous occlusion plethysmography as described in detail before. During the measurements a wrist cuff excluded the hand circulation. Blood pressure was measured intra-arterially using a Hewlett Packard 7820SC monitor while heart rate was derived from the ECG. Basal measurements of blood pressure, heart rate, and FBF were obtained 15 minutes after insertion of the arterial catheter. Following another 15 minutes, the first dose-response curve of BNP was determined, with FBF being recorded continuously from 2 minutes before until the end of the infusion of this peptide. The mean value of the last minute of each dose was used for analysis. Infusion of TEA and/or L-NMMA always started 5 minutes before the subsequent BNP dose-response curve. As described before, TEA does not influence baseline FBF. Furthermore, vasodilatation induced by SNP is not inhibited by TEA, indicating that TEA has no inhibitory effect on endothelium-independent vasodilation. However, because FBF is reduced by L-NMMA, we added sodium nitroprusside (SNP) to the L-NMMA infusion in order to maintain FBF at its original level ("NO-clamp" technique) before starting the next
BNP infusion. The dose of co-infused SNP was titrated individually and held constant during infusion of BNP.

All signals were stored on the hard disk of a personal computer by means of a custom-built data acquisition system (Instrumental Services, Maastricht University, The Netherlands).

Drugs

On the day of use, TEA was reconstituted from a sterile stock powder (Sigma) diluted in 0.9% NaCl to a concentration of 1 mg/ml. Further dilutions were prepared from ampules in 5% glucose. All solutions were freshly prepared on the day of experiment and stored at 4°C until use. BNP and L-NMMA were obtained from Clinalfa (Ethifarma Nederland BV, The Netherlands).

Assays

BNP, CNP and cGMP were measured by means of radioimmunoassay (Peninsula Laboratories Inc. RIK 9086 and RIK 9030, and IBL Hamburg RE 29071, respectively). Before the radioimmunoassay procedure plasma samples of both BNP and CNP were acidified and extracted using a Sep-Pak C18 column (Waters-Millipore). In our hands, the intra- and interassay variability are 6.2% and 10.8% respectively for BNP, 6.8% and 11.3% for CNP and 4.3% and 8.6% for cGMP. The antisera for BNP and CNP did not crossreact with the other peptide. All samples from the same individual were assayed in a single run.

Statistics and calculations

For each measurement of FBF, we calculated the ratio of the infused and the contralateral arm was calculated. This ratio corrects for all systemic factors, which may affect blood flow in both arms and is stable during the day. Moreover, this ensures that the direct effects of locally infused substances on forearm blood flow can be assessed. For each separate dose of drug, we determined the individual percentage change in FBF ratio (relative to pre-infusion values). In addition, the areas under the dose-response curves (AUC) were calculated for each individual to avoid the statistical problem of repeated measurements. Net forearm increases of CNP and cGMP were calculated according to the method of Nakamura et al. as the difference between plasma levels of CNP or cGMP at baseline and after the maximum dose of BNP, multiplied by the corresponding change in FBF.

Data are presented as medians with interquartile ranges (IQR). Friedman’s test (non-parametric two-way ANOVA) was used for analysis of multiple related samples (within one visit) and Wilcoxon paired sign test for paired analysis (between visits and within one visit). P values below 0.05 denote statistical significance.
Results

The median body weight of the participants was 68 (IQR: 67-69) kg and their height 1.80 (1.76-1.82) m. This represents a median body mass index (BMI) of 20.5 (20.0-21.9) kg/m².

Blood pressure and heart rate

Mean arterial pressure (MAP) and heart rate (HR) did not change during any of the studies (Table 6.1), indicating that the local infusions of BNP or inhibitors had no systemic hemodynamic effects. Furthermore, there were no differences in MAP and HR between both visits.

Effect of BNP alone on forearm blood flow

Baseline FBF values of the infused arm [2.2 (2.0-3.3) ml/dl/min at visit 1 and 2.2 (1.9-3.1) ml/dl/min at visit 2; p=0.208], the contralateral arm [2.2 (1.9-2.6) ml/dl/min and 2.1 (1.8-2.5) ml/dl/min, respectively; p=0.594], and the FBF-ratio [1.3 (0.9-1.6) ml/dl/min and 1.2 (0.9-1.3) ml/dl/min, respectively; p=0.263] were similar on both days. BNP alone induced a dose-dependent dilatation, which was comparable on the two days (Figure 6.1). The absolute FBF of the infused arm increased to 9.1 (8.0-11.5) ml/dl/min at the highest dose (p=0.005) at visit 1, and to 9.4 (8.1-11.5) ml/dl/min on visit 2 (p=0.005). Absolute FBF values of the contralateral arm did not change during BNP infusion.

At the end of infusion, the four doses of BNP had increased the FBF ratio by 216% (164-372), and 207% (169-454), respectively, relative to baseline (p<0.001 for both visits; Figure 6.1). The medians of the area under the dose-response curves (AUC) were 7602 (5102-13580) and 7192 (5831-17145) units respectively.

Table 6.1. Heart Rate and Mean Arterial Pressure of the Subjects at the Beginning and End of the Two Visits

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate (beats x min⁻¹)</th>
<th>Mean Arterial Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>Visit 1</td>
<td>56 (53-61)</td>
<td>59 (54-66)</td>
</tr>
<tr>
<td>Visit 2</td>
<td>55 (50-62)</td>
<td>60 (52-64)</td>
</tr>
</tbody>
</table>

Blood pressure was measured intra-arterially. Values are presented as median and interquartile range.

Effect of inhibitors on BNP-induced vasodilatation

At the start of all combined infusions the FBF ratios [1.2 (1.0-1.5), 1.2 (1.0-1.3), and 1.1 (0.9-1.5) respectively] were comparable to baseline values (p=0.927).
TEA. The absolute FBF value in the infused arm was 2.5 (2.3-3.8) ml/dl/min and 2.1 (1.8-3.0) ml/dl/min in the contralateral arm before infusion. In combination with TEA, the four doses of BNP increased the FBF ratio by 32% (16-49), 70% (30-111), 105% (76-152), and 150% (79-294) respectively (p<0.001; Figure 6.1). When the maximal effect of BNP alone is set at 100%, TEA reduced the vasodilatory effect of BNP by 45% (20-58) at the highest dose of 64 pmol/dl/min (p=0.005). The median AUC for BNP with TEA was significantly smaller than with BNP alone (p=0.009; 6. 2).

![Graph showing vasoreactivity of forearm (muscle) vasculature to local intra-arterial infusions of BNP with and without inhibitor(s), expressed as the area under the curve (AUC). Data are presented as median and interquartile range. In all conditions BNP caused significant dilatation (p<0.05). * p<0.05 versus BNP infusion alone. † p<0.005 versus BNP+TEA.](image)

L-NMMA. The absolute FBF value in the infused arm was 3.0 (2.5-4.1) ml/dl/min and 3.0 (2.0-3.5) ml/dl/min in the contralateral arm before infusion. In combination with L-NMMA, the four doses of BNP increased the FBF ratio by 11% (-6-71), 39% (2-77), 74% (15-121), and 128% (34-190) respectively (p<0.001; Figure 6.1). This corresponds to a reduction of maximal BNP-induced dilatation by 63% (33-82) at the highest dose of 64 pmol/dl/min (p=0.008). The median AUC for BNP with L-NMMA was significantly smaller than that of BNP alone (p=0.038), but not different from the AUC for BNP combined with TEA (p=0.314; Figure 6.2).

Combination of TEA and L-NMMA. The absolute FBF value in the infused arm was 3.3 (2.5-4.2) ml/dl/min and 2.8 (2.3-3.2) ml/dl/min in the contralateral arm before infusion. When combined with both TEA and L-NMMA, the four doses of BNP increased the FBF ratio by 3% (-26-21), 9% (-11-36), 28% (18-57), and 43% (29-113) respectively (p<0.001; Figure 6.1). This corresponds to a reduction of maximal BNP-induced dilatation by 77% (69-87) at the highest dose of 64 pmol/dl/min (p=0.005). The median AUC for BNP in combination with TEA and L-NMMA was significantly smaller than that of BNP alone (p=0.005) and that of BNP in combination with TEA (p=0.005; Figure 6.2). No significant difference was observed between the effects of BNP in
combination with L-NMMA and those of BNP in combination with both L-
NMMA and TEA (p=0.214).

Plasma levels of BNP and CNP, and cGMP

Forearm intra-arterial infusion of BNP caused the expected increase in
venous BNP levels (p<0.01). Baseline plasma BNP levels were 29 (25-
35) pg/ml and 26 (24-35) pg/ml respectively, with no difference between
both visits. Plasma BNP levels reached peak values of 3287 (2928-5398)
pg/ml, 3813 (2862-5441) pg/ml, 3910 (3379-4925) pg/ml, 5066
(3933-7377) pg/ml, and 3270 (2126-3270) pg/ml, respectively after
infusion of BNP alone, BNP with TEA, BNP with L-NMMA, and BNP in
combination with both inhibitors. This rise in venous BNP levels was
associated with an increase in venous plasma levels of CNP and cGMP.
The plasma CNP level was 3.2 (2.1-4.3) pg/ml at baseline and reached
values of 7.1 (5.9-9.4) pg/ml, and 7.5 (4.8-10.9) pg/ml, respectively
after infusion of BNP alone, and BNP in combination with L-NMMA.
Although the net calculated CNP increases rose significantly both after
infusion of BNP alone and after BNP with L-NMMA, this increase did not
differ between both conditions: 12.2 (7.0-16.5) fmol/dl/min versus 10.4
(0.7-15.1) fmol/dl/min.
The plasma cGMP level was 6.4 (5.8-8.7) pmol/ml at baseline and
reached peak values of 13.9 (12.1-18.6) pmol/ml, 23.8 (20.0-28.9)
pg/ml, 22.2 (19.2-30.0) pmol/ml, and 34.9 (24.1-41.4) pmol/ml,
respectively after infusion of BNP alone, BNP with L-NMMA, BNP with
TEA, and BNP in combination with both inhibitors. Net calculated cGMP
increase also rose significantly after local infusion of BNP in all
conditions (Figure 6.3).

![Figure 6.3](image)

Figure 6.3 Net forearm increase in cGMP induced by local intra-arterial infusions of
BNP with and without coinfusion of inhibitor(s). Data are presented as
median and interquartile range. In all conditions net forearm CNP increase
rose significantly (p<0.05). * p<0.05 versus BNP infusion alone.

Coinfusion of BNP and TEA did not change the cGMP net increase as
compared to that caused by BNP alone: 47.8 (39.2-65.4) pmol/dl/min
versus 37.5 (21.9-58.1) pmol/dl/min. In contrast, BNP was less potent in increasing cGMP with coinfusion of either L-NMMA or L-NMMA and TEA: 33.2 (18.3-47.0) pmol/dl/min and 15.7 (12.2-42.4) pmol/dl/min (p=0.013 and p=0.007 respectively).

Discussion

The present study shows that local BNP infusion in healthy men induces a dose-dependent vasodilatation, which is significantly attenuated by both potassium-channel inhibition and NO-synthase inhibition. In addition, we found that NO-synthase blockade reduced the BNP-related increase of cGMP, but not of CNP. These findings support the notion that BNP exerts its biological action, at least in part, through stimulation of cGMP and CNP production and opening of potassium channels on the one hand and via NO production on the other. Since no changes in blood pressure and heart rate were apparent, it is unlikely that the effects on forearm blood flow were related to systemic hemodynamic effects of the agents.

At the highest doses, intra-arterial infusion of BNP caused a 100-fold increase in venous plasma BNP levels on both visits. These BNP plasma levels can be considered to be in the pharmacological range, although in heart failure patients it has been shown that plasma BNP levels may be as much as 25-180 times higher as compared to healthy subjects. In the present study, however, vasodilatation already ensued with a dose of 16 pmol/dl/min during which plasma levels must have been within the pathophysiological range.

BNP is thought to induce vasodilatation by increasing intracellular levels of cGMP, which in turn stimulates Ca\(^{2+}\)-activated, and ATP-sensitive potassium channels, thus causing hyperpolarisation.\(^5\)\(^,\)\(^10\) TEA antagonises different kinds of potassium channels with varying degrees of potency, but has been shown to selectively block K\(^{+}\)\(\text{Ca}^{2+}\)-channels in arterial smooth muscle at concentrations below 1 mmol/l.\(^17\) In the present study, we administered TEA intra-arterially at an infusion rate of 0.1 mg/dl/min, which correlates with a calculated local plasma concentration of 0.5 mmol/l.\(^20\) Since TEA did not abolish the BNP-induced dilatation, but only reduced it by approximately 50%, our data indicate that besides opening of K\(^{+}\)\(\text{Ca}^{2+}\)-channels, other dilator pathways must be involved in the vascular effects of BNP. One such pathway may be related to NO, as we found that L-NMMA also significantly reduced BNP-induced vasodilatation. This corroborates data from cellular and animal studies, which also have shown that natriuretic peptides are able to stimulate NO production.\(^1\)\(^7\)\(^,\)\(^28\)\(^,\)\(^29\) It is not clear, however, whether NO production is stimulated via the NPR-A or via an alternative route. In any case, the present study does not provide evidence that NO release
induced by BNP would involve stimulation of the NPR-A. Moreover, others have shown that the natriuretic peptide clearance receptor (NPR-C) is more likely to mediate the NO response.29 The possibility that besides NO alternative pathways (e.g. prostaglandins) are involved in BNP-induced vasodilatation cannot be ruled out, since the combination of NO-synthase and K^+_{Ca^{2+}}-channel blockade did not completely prevent the rise in flow.17 Nevertheless, the fact that the combination of TEA and L-NMMA attenuated BNP-induced vasodilatation even more than TEA alone, does support the hypothesis that both pathways (NPR-A activation and stimulation of NO) act in parallel. It may even be that the effect on NO is quantitatively more important since the combination of TEA and L-NMMA did not have a greater effect than L-NMMA alone.

Our data further show that BNP induces CNP release. CNP is thought to be one of the endothelium derived hyperpolarizing factors (EDHF's), which serve as an autocrine/paracrine endothelium-derived vaso-regulatory system.30 CNP production in vascular endothelial cells probably plays an important role in the NPR-A / potassium channel pathway. For instance, Nazario et al. demonstrated in cultured endothelial cells that BNP as well as ANP can stimulate CNP production through a guanylate cyclase receptor. CNP stimulates the natriuretic peptide receptor B (NPR-B) on adjacent vascular smooth muscle cells, thereby leading to increases in cellular cGMP, activation of potassium channels and vasodilatation.13,14

The CNP data from the present study provide additional support for our hypothesis that no interaction exists between NPR-A activation and stimulation of NO by BNP. Venous CNP levels increased significantly after local infusion of BNP alone as well as after BNP in combination with L-NMMA, there being no difference between both conditions. In as much as increased CNP "spillover" reflects NPR-A activation, it follows that the two pathways of action of BNP are independent from each other. Moreover, both in vitro and human studies have shown that CNP itself does not stimulate NO production.11,31 We do realise, however, that the net increase in CNP release can be influenced by (receptor) clearance, and that the only way to quantify CNP production correctly is using tracer-labelled CNP infusions. Therefore, some caution in the interpretation of our results remains warranted. With this proviso, Figure 6.4 gives a schematic account of how the various results from this study may be put together.

In disease states, such as hypertension and congestive heart failure, several mechanisms may be altered and dysfunctional, including the sensitivity of BNP receptors, the status of potassium channels, and the production of NO. Hence, one cannot directly extrapolate our findings to pathophysiological states. Furthermore, we were not able to study the mechanisms of BNP-induced vasodilatation in greater detail since NPR antagonists and/or cGMP inhibitors are not available for human use.
Likewise, we could not differentiate between endothelium-dependent and independent mechanisms. We do not know, therefore, to what extent potassium channels in the endothelial cells contribute to the actions of BNP, over and above those in smooth muscle cells.

Figure 6.4 Simplified hypothetic overview of the vascular actions of BNP in humans. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; NO, nitric oxide; cGMP, cyclic GMP; A, guanylate cyclase bound natriuretic peptide receptor A; B, natriuretic peptide receptor B; EC, endothelial cell; VSMC, vascular smooth muscle cell; SG, soluble guanylate cyclase; K\(^+\), Ca\(^{2+}\)-activated potassium channel.

Another limitation of the present study is that we did not include a control group in which repeated BNP infusions were given without TEA or L-NMMA. However, we have demonstrated previously that forearm vascular responses to BNP are remarkably reproducible upon repeated administration of the peptide and within the time frame of our studies no tachyphylaxis is to be expected. Another limitation is that we could not assess the effects of L-NMMA alone and L-NMMA in combination with TEA on separate days. Since the latter would have required a third intracutaneous study, we preferred to combine these experiments on one day.

In conclusion, the present data demonstrate that BNP induces vasodilatation in healthy men via at least two mechanisms: one involving hyperpolarisation via opening of K\(^+\)-Ca\(^{2+}\)-channels and one via stimulation of NO production. Furthermore, we have shown that BNP stimulates net CNP increase.
References


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Chapter 7

Review
"Brain natriuretic peptide in human physiology; compensating mechanism in vascular disease?"

K van der Zander, PW de Leeuw
Introduction

Brain natriuretic peptide (BNP), a member of the family of natriuretic peptides, receives increasing attention as a potential marker of cardiovascular disease. For instance, left ventricular hypertrophy (LVH) or left ventricular dysfunction are associated with increased BNP levels, and in patients with coronary insufficiency or heart failure, BNP has prognostic significance. However, despite many investigations, the hemodynamic effects of BNP in man remain elusive. Due to differences in design, doses of BNP employed, and infusion time, data from the literature are difficult to compare.

The aim of this review is to summarize our current understanding of BNP, and discuss its regulation, its mechanisms of action and its role in pathophysiological states such as hypertension and heart failure.

Regulation of plasma BNP

Although BNP was first identified in the porcine brain, this peptide is produced mainly in the cardiac ventricle and is secreted through the coronary sinus from the heart. The amino acid sequences of BNP vary from species to species. In contrast to porcine BNP and rat BNP consisting of 26 and 45 amino acid residues respectively, in humans the major circulating form of BNP consists of 32 amino acids. Human BNP has a characteristic ring structure, formed by an intramolecular disulfide bridge, and amino- and carboxyl-terminal tails. The main stimulus for the acute release of BNP is wall stretch in response to chronic volume and pressure overload. There are also non-hemodynamic components involved, whereas in the ventricles the level of BNP expression seems to be partly dependent upon neuroendocrine mechanisms. For instance, α1-adrenergic stimulation with phenylephrine (PE) results in increases in both BNP secretion and BNP gene expression, whereas stretch and endothelin-1 (ET-1) induce a marked increase in BNP gene expression alone.

Normal plasma levels of BNP vary from 4 to 35 pg/ml (or 1.2 to 10 pmol/l). These levels rise with age and are higher in females than in males. In man, intravascular saline loading has no effect on plasma BNP concentrations, while an increase in dietary sodium stimulates BNP levels. Furthermore, plasma levels of BNP respond to posture-induced changes in central volume and fall, for instance, when the individual moves from the supine to the sitting position. The observation that plasma levels of BNP are elevated in disease states associated with volume overload, such as heart failure and some forms of hypertension,
are in line with the hypothesis that BNP levels reflect one's extracellular volume. Hence, BNP may be used as a marker for left ventricular dysfunction or left ventricular hypertrophy (LVH), when these conditions are associated with disturbed volume control.

Mechanisms of action

Molecular cloning techniques have revealed three subtypes of natriuretic peptide receptors (NPR), named NPR-A, NPR-B, and NPR-C.29 In vitro studies suggest that BNP and ANP exert their vascular effects through the NPR-A. This membrane-bound guanylate cyclase receptor is located both on endothelial and vascular smooth muscle cells.27,28 Activation of this receptor generates cGMP, which as second messenger activates Ca++-activated and ATP-sensitive potassium (K+) Channels, thus promoting vasorelaxation.29,30 Furthermore, Nazario et al. demonstrated in cultured endothelial cells that BNP as well as ANP can stimulate c-type natriuretic peptide (CNP) production in endothelial cells through the guanylate cyclase receptor.27 CNP stimulates the NPR-B on adjacent vascular smooth muscle cells, thereby leading to increases in cellular cGMP, activation of potassium channels and vasodilatation.26 There is also some evidence from cellular and animal studies that natriuretic peptides are able to stimulate nitric oxide (NO) production11,33, which is probably mediated by the natriuretic peptide clearance receptor (NPR-C). The NPR-C decreases cAMP levels by adenyl cyclase inhibition through an inhibitory guanine nucleotide-regulating protein.34 This suggests that besides being a clearance receptor, NPR-C is also biologically active. Natriuretic peptides are not only cleared by the NPR-C, but circulating ANP, BNP and CNP are also quickly metabolised and inactivated by the specific enzyme neutral endopeptidase (EC 24.11). However, BNP has a different plasma half-life than ANP. In human beings, the half-life of BNP has been reported to be approximately 20 minutes as opposed to 3 minutes for ANP.3,35

In disease states it is possible that the NPR's are down-regulated for natriuretic peptides. Indeed, several studies have indicated that long-term exposure to exogenous ANP decreased receptor density and reduced responsiveness in terms of intracellular accumulation of cGMP in cultured vascular smooth muscle cells.16,37 This inhibition may also occur during exposure to BNP.

Effects of BNP on the cardiovascular system

Acute infusion of a pharmacological dose of BNP into normal subjects may lead to a rapid and sustained reduction in mean arterial blood
pressure. Mechanisms by which BNP produces this effect include diminished stroke volume, a reduction in systemic vascular resistance, and increased diuresis with ensuing intravascular volume depletion. Cardiac output and heart rate increase after pharmacological infusion of BNP, probably due to baroreceptor-mediated sympathetic activation in response to the fall in arterial blood pressure. However, like in chapter 2, in most studies in which (patho)physiological doses of BNP were infused into healthy volunteers, no effects on mean arterial blood pressure were observed. \textsuperscript{18-19,21,22} In studies from Jensen et al.\textsuperscript{14,15} mean arterial pressure even slightly increased during low-dose BNP infusion. These investigations assessed the hemodynamic effects of placebo and different low doses of BNP infusion. However, the observed rise in MAP may be an aspecific phenomenon as this was also seen in the placebo group and no significant differences were found between placebo and BNP groups.\textsuperscript{14} Aside from the study by Holmes et al.\textsuperscript{19}, no changes in heart rate have been found after infusion of BNP in doses not exceeding 4 pmol/kg/min.\textsuperscript{14,18,21,22} The incidental finding of an increase in heart rate can probably be explained by the influence of posture as Holmes et al.\textsuperscript{10} measured in sitting position in contrast to the other investigators. This explanation is supported by our own observation, described in chapter 2, that BNP induced a significant fall in blood pressure and a rapid increase in heart rate only when our subjects switched from supine to sitting position. The observed decrease in plasma BNP in response to posture-induced changes in central volume could even be a compensating mechanism to prevent orthostatic hypertension. Furthermore, infusion of low doses of BNP into normal subjects does not reduce peripheral vascular resistance or cardiac output, while it decreases stroke volume.\textsuperscript{16,18} These observations suggest that BNP does not primarily affect the arterial vascular bed, but rather reduces preload, possibly by lowering venous return. The latter may, of course, be secondary to reduced intravascular filling, although not necessarily so. Similarly as in healthy subjects, BNP infusion in doses not exceeding 4 pmol/kg/min does not elicit any appreciable modifications of systemic hemodynamics and left ventricular performance either in patients with hypertension, who already have increased BNP plasma levels.\textsuperscript{38,39} When BNP was administered in higher doses, significant blood pressure-lowering effects were observed, which were two- to threefold those of ANP.\textsuperscript{40} In patients with heart failure, BNP administered in lower doses (i.e. below 4 pmol/kg/min) infusion caused significant falls in blood pressure and left ventricular filling pressure, without concomitant changes in cardiac output and heart rate.\textsuperscript{41,42} Just as in healthy subjects, Jensen et al. again found quite unexpectedly that the mean arterial blood pressure increased slightly in patients with CHF during BNP infusion.\textsuperscript{15} BNP in pharmacological doses (30 pmol/kg/min) has a beneficial effect on left ventricular function similar to ANP by decreasing systemic vascular resistance and also by increasing stroke volume index.\textsuperscript{28,42,47}
Taken together, in healthy subjects and hypertensive patients, low doses BNP has no appreciable effects on the cardiovascular system, but in pharmacological doses the peptide lowers blood pressure. On the other hand, both low and pharmacological dose of BNP have favourable effects on cardiovascular and renal hemodynamics in patients with heart failure. This can probably be explained by the fact that baseline BNP plasma levels are higher in patients with heart failure compared to healthy subjects and even more increased than in hypertensive patients.\textsuperscript{3,6,25}

**Renal actions of BNP**

Although systemic infusion of BNP stimulates natriuresis and diuresis\textsuperscript{14,21}, the hemodynamic effects of BNP on the renal vasculature are not clear. Most, but not all, studies in healthy humans have reported that BNP infusion increases glomerular filtration rate\textsuperscript{14,15,16,20}, whereas renal plasma flow decreases\textsuperscript{14}, increases\textsuperscript{15} or remains unchanged.\textsuperscript{15,17,21} One possible explanation for the variations in the renal effects of BNP may be that different BNP levels were reached during the experiments. Concurrent changes in GFR and RPF only occurred when the dose of BNP exceeded 2 pmol/kg/min.\textsuperscript{14,15,18,20} With lower doses of BNP, several authors\textsuperscript{17,19,21} observed a natriuretic and diuretic effect of BNP in the absence of changes in RPF and GFR. Although we have used a dose of BNP similar to that in the studies of Jensen et al.\textsuperscript{14} and La Villa et al.\textsuperscript{18} in chapter 2 and 4, we could not demonstrate any change in RPF, while GFR increased. However, some caution is warranted in this respect. For instance, Jensen et al.\textsuperscript{14} did not compare placebo and BNP in the same subjects. This may have introduced at least some bias. The difference between our findings and those of La Villa et al.\textsuperscript{18} can probably be explained by the small number of (younger) subjects and the lower salt intake in their study as compared to ours. Jensen et al.\textsuperscript{14,15} have demonstrated by lithium clearance that proximal reabsorption of sodium may be reduced by BNP. These observations suggest that BNP has a direct effect on sodium handling within the kidney even in lower doses and without any changes in GFR or RPF.

In hypertensive patients BNP infusion also exerted a marked natriuresis and diuresis. Indeed, as in normal subjects concurrent changes in GFR only occurred when the dose of BNP exceeded 2 pmol/kg/min.\textsuperscript{38,39} Infusion of BNP induced also natriuresis in patients with heart failure, but the natriuretic effect was impaired compared with healthy subjects and hypertensive patients.\textsuperscript{15,20,41-43}, most likely due to reduced responsiveness of the distal part of the nephron.\textsuperscript{15}
Local effects

After studies that showed that human BNP could relax human arteries\textsuperscript{44}, we (in chapter 5) and others have demonstrated an in vivo dose-dependent vasodilatation of the forearm vasculature of healthy subjects.\textsuperscript{45,46} However, in this respect BNP is less potent in comparison with ANP.\textsuperscript{45} The difference in effect may be related to a difference in affinity for the NPR-A\textsuperscript{26}, but there is data in literature that does not support this concept. For instance, human BNP and ANP are equipotent in relaxing isolated preconstricted human arteries.\textsuperscript{44} Also, studies in cultured endothelial cells have shown that BNP and ANP have similar dissociation constants and maximal binding capacities for the NPR-A, and both peptides are equipotent in stimulation of endothelial cGMP production.\textsuperscript{47,48} BNP even induces a 20-fold greater release of CNP by endothelial cells than ANP.\textsuperscript{27} Finally, the forearm blood flow responses to BNP were significantly lower in heart failure patients, and the difference in vasodilatation between BNP and ANP as observed in the healthy subjects was not present in patients with heart failure.\textsuperscript{46} To the best of our knowledge, no forearm study with BNP has been performed in patients with hypertension. In conclusion, these observations showed firstly that BNP and ANP act differently, and secondly, that more mechanisms must be involved than NPR-A alone, and thirdly, that the vasorelaxant capability of natriuretic peptides is impaired in patients with heart failure. Our findings from chapter 6 support the notion that BNP exerts its biological action, at least in part, not only through stimulation of cGMP and CNP production and opening of potassium channels, but also via NO production.\textsuperscript{49} These different pathways may account for a compensatory mechanism when endothelial function is impaired. In case of downregulation of the NPR-A, production of NO will be more important. On the other hand, in case of decreased availability of NO, stimulation of the NPR-A augments cGMP generation. Recent studies have already emphasised the importance of BNP and NO, for example in heart failure\textsuperscript{50}, but our results indicate that there is an interaction between the two. Further research is needed to elucidate how BNP stimulates NO production and if more pathways are involved in the biological effects of BNP.

Current clinical interest and future perspectives

Data from the literature of the last decade clearly delineate brain natriuretic peptide (BNP) as a cardiac hormone of clinical interest in diagnosis, prognosis and treatment of patients with heart failure as well as in patients with asymptomatic left ventricular systolic dysfunction. Basal plasma levels of BNP are used for classifying the degree of heart
failure, which appears to be more objective than the NYHA (New York Heart Association) classification. A cut point of 100 pg/mL discriminates patients with congestive heart failure from those without congestive heart failure. Although both BNP and ANP increase in patients with heart failure, BNP plasma levels are more proportional to the functional severity of heart failure and frequently surpass ANP levels. Furthermore, it has been demonstrated that BNP can fall back to normal levels in well-compensated patients despite persisting significant systolic dysfunction. This suggests that BNP assays may be helpful for monitoring adequacy of therapy, but that BNP assays will have limited utility in the diagnosis of cardiac impairment once anti-failure therapy has been well established and symptoms have been abolished. Measurement of BNP may also be a screening tool for left ventricular dysfunction. However, in the large community-based sample of the Framingham heart study, the performance of BNP measurements for detection of elevated left ventricular mass and systolic dysfunction was suboptimal, suggesting limited usefulness of natriuretic peptides as mass screening tools. However, to consider natriuretic peptide levels only as a general and functional indicator of cardiac structural disease, without recalling that atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are powerful hormones, may lead to underestimation of the physiological role these peptides play in healthy subjects as well as in patients with heart failure. In this view, a peptide currently manufactured by recombinant DNA technology and identical to endogenous BNP (nesiritide), has been evaluated in clinical trials as a therapeutic option. The drug produces a prompt fall in systemic vascular resistance and pulmonary capillary wedge pressure, associated with rapid clinical improvement in decompensated heart failure. Nesiritide was even found to be more effective and better tolerated than the venous vasodilator nitroglycerin. This thesis dealt mostly with healthy subjects to determine the specific hemodynamic effects of BNP on different target organs in an attempt to reveal -in vivo- the underlying mechanisms of action. On the basis of the results we can now hypothesise how BNP can play a key role in cardiovascular disease states. Our findings in chapter 5 that BNP is a mild dilator compared to equimolar doses of ANP, together with the lack of variation in renal blood flow after BNP infusion as described in chapter 2 and 3, led us to hypothesise that the net effect of BNP is dependent on the balance between vasodilating and vasoconstrictor forces. An observation that supports this hypothesis is a study that showed that infusions of BNP in normal conscious dogs caused dose-related, reversible mesenteric vasoconstriction. Vasoconstrictor actions may counterbalance the other hemodynamic actions of BNP, thereby preferentially redistributing blood flow to prevent precipitous falls in arterial blood pressure. When arterial and venous filling pressures fall due to pharmacological doses of BNP, reflex sympathetic stimulation is to
be expected. However, after low-dose BNP infusion a sympathoinhibitory effect of BNP has been demonstrated, whereas high dose BNP did not change systemic and cardiac sympathetic nervous activity. Furthermore, autonomic ganglion blockade with pentolinium did not prevent the BNP-induced mesenteric vasoconstriction. This suggests some direct vasoconstrictor actions of BNP on the vasculature, independent of the nervous system. However, changes of body position during administration of BNP does activate the baroreceptor reflex as we observed in chapter 2. When we combine our observations on the systemic and the peripheral vasculature, it would seem that the most probably site of action for BNP is the venous system where it may increase the 'unstressed' volume. This would explain, at least in part, the beneficial effects of this peptide in patients with heart failure.

Another explanation for the vasoconstrictor potency of BNP could be that endothelin-1 (ET-1) acts as a possible mediator of the direct vascular actions of BNP. Indeed, it has been demonstrated before that a close relationship exists between BNP and ET-1 production at least within the kidney. Apart from being a potent vasoconstrictor, ET-1 exhibits intrarenal natriuretic activity independently of changes in filtered load. The role of ET-1 in the kidney may even be dissociated from circulating ET-1. In fact, De Feo et al. demonstrated that systemic infusion of BNP is accompanied by increased urinary excretion of ET-1, cGMP and sodium, without changes in plasma ET-1. ET-1 acts in an autocrine and paracrine manner on two subtypes of ET receptors, termed ET-A and ET-B. These receptors are located on vascular smooth muscle cells and binding of ET-1 to these sites results in sustained vasoconstriction. However, ET-B receptors are also present on endothelial cells where their activation leads to production of NO and vasodilator prostanoids, and subsequent vasodilatation. In chapter 6, we demonstrated that BNP acts partly via the production of NO. Thus, it may be that the close relationship between BNP and ET-1 production in the kidney means that ET-1 mediates (part of) the effects of BNP via the ET-B receptor. If this were the case, this would introduce an interesting novel interaction for therapeutical interventions. Indeed, currently a number of drugs are under investigation, which either inhibit the enzyme that degrades natriuretic peptides (neutral endopeptidase inhibitors) or block endothelin receptors. Both types of agents are evaluated for their potential in the treatment of cardiovascular disease. Concurrent administration of a neutral endopeptidase inhibitor and a selective ET-A receptor antagonist would greatly enhance BNP's effect on the kidney if ET-B receptors were to mediate these effects. As described in the study of chapter 4, however, ET-B receptor blockade did not influence the BNP-induced renal responses (enhanced GFR and natriuresis). BNP could increase GFR in three possible ways, namely by preglomerular vasodilatation, by post-glomerular vasoconstriction, and/or by changing filtration surface area. The first possibility, pre-glomerular
vasodilatation, is not very likely to have occurred since we did not notice any changes in ERPF. Of course, we cannot exclude that BNP augments filtration surface area, but presently there are no good tools to investigate this in an unbiased way. For the time being, therefore, we consider post-glomerular vasoconstriction the most likely mechanism of the BNP-induced rise in GFR. It is still possible that ET-1 mediates the BNP-induced effects via the ET-A receptor, since experiments conducted on anaesthetised dogs with ET-A and ET-B specific antagonists, have shown that ET-B receptors predominantly account for diuresis and natriuresis, whilst the ET-A receptor is largely responsible for renal vasoconstriction.  

In conclusion, stimulation of BNP may be a compensating mechanism in vascular diseases, but endogenous BNP does not seem to be able to fully compensate for volume overload in hypertension and heart failure. The therapeutic benefits of BNP infusion are particular evident clear in patients with heart failure, although the hemodynamic effects of BNP appear to be dependent upon the used dose. Therefore, more research focussed on the specific mechanisms of action of BNP, and especially on the possible vasoconstrictor properties of BNP, is needed.
References


Chapter 8

Summary
Summary

This thesis describes the local and systemic effects of BNP on different target organs (heart, kidney, forearm vasculature, and finger skin and conjunctival microcirculation) and which mechanisms play a role in these effects.

After a short general introduction in chapter 1, chapter 2 the hemodynamic and renal effects of low-dose BNP infusion in healthy subjects is described. BNP decreased stroke volume with a tendency to decrease cardiac output, possibly by lowering venous return. However, BNP did not affect the microvasculature, at least not in skin or conjunctiva. Infusion of BNP increased natriuresis, diuresis, GFR, filtration fraction, and filtered load of sodium, while ERPF did not change. We concluded that BNP has predominantly central and renal hemodynamic effects, while it does not influence peripheral micro-circulation.

The direct renal effects of BNP in hypertensive patients is investigated in the study described in chapter 3. Intrarenal BNP infusion did not induce significant changes in renal blood flow, despite increases in circulating levels of cGMP. In addition, we did not find any BNP-related changes in the cGMP gradient across the kidney, in the secretion of active renin and in creatinine extraction. These observations suggest a primarily extrarenal target of BNP. Another explanation could be that BNP, besides pre-glomerular vasodilatation, induces post-glomerular vasoconstriction, with the net effect that RPF remains constant.

Chapter 4 describes whether renal endothelin-1 could possibly mediate (part of) the effects of BNP via the ET-B receptor in healthy subjects. Apart from being a potent vasoconstrictor, endothelin-1 exhibits natriuretic activity independently from changes in filtered load. This effect of endothelin-1 is exerted via the endothelin-1 B receptor. However, while our study showed that selective ET-B receptor antagonism by itself decreased renal sodium excretion, it had no effect on the BNP-induced natriuresis and rise in glomerular filtration rate. Further studies are needed to elucidate a possible role of endotheline-1 (acting via the ET-A receptor) in mediating the renal effects of BNP.

The study in chapter 5 concerns the local vasoactive effects of BNP as compared to those of ANP in the forearm vasculature of healthy subjects. The results of this study showed that both BNP and ANP induce a dose-dependent vasodilatation, which is reproducible in time. In addition, we found that the degree of vasodilatation induced by BNP is significantly
less than those following equimolar doses of ANP. Since both BNP and ANP act through the same receptor, these findings suggest that more pathways are involved in the biological action of BNP.

The mechanisms of action of BNP in the human forearm were further investigated in chapter 6. This study demonstrated that BNP, besides activation of natriuretic peptide receptor A and thereby opening of potassium-channels, also stimulated nitric oxide (NO) production to induce vasodilatation in the human forearm. Furthermore, we showed that BNP stimulates cGMP and c-type natriuretic peptide production.

In chapter 7 the results of the above-mentioned studies were discussed in the context of current literature. Finally, the clinical relevance of our results for the treatment and/or prevention of hypertension and/or heart failure is described.
Chapter 9

Samenvatting
Samenvatting

Dit proefschrift beschrijft de lokale en systemische effecten van BNP op verschillende doelorganen (hart, nieren, onderarmbloedvaten, en de kleine bloedvaten van oog en nagelplooi) en welke werkingsmechanismen daaraan ten grondslag liggen.

Na een korte introductie in hoofdstuk 1, worden in hoofdstuk 2 de hernadynamische en renale effecten van infusie van een lage dosis BNP ten opzichte van placebo bij gezonde proefpersonen beschreven. BNP blijkt de hoeveelheid bloed die het hart uitpompt, vermoedelijk als gevolg van een verlaagd aanbod van bloed vanuit de circulatie, te verlagen. BNP heeft echter geen effect op de kleine bloedvaten van oog en nagelplooi. De nieren gaan, door de infusie van BNP, meer water en zout uitscheiden zonder dat ze meer bloed van het hart aangeleverd krijgen. De nierdoorbloeding verandert niet door BNP, maar de hoeveelheid bloed die wordt gefilterd door de nieren stijgt. We concluderen dat BNP alleen effect lijkt te hebben op de centrale en renale hemodynamiek, zonder de perifere hemodynamiek te beïnvloeden.

De effecten van BNP op de nier worden op lokaal niveau verder onderzocht bij mensen met hoge bloeddruk, zoals beschreven in hoofdstuk 3. We kunnen geen direct effect aantonen van BNP op de nierdoorbloeding of filtratie, ondanks een stijging van de second messenger van BNP (cGMP). Dit wil zeggen dat de nieren het effect van BNP op de rest van de circulatie nodig hebben om zout- en wateruitscheiding te kunnen bewerkstelligen. Een andere verklaring zou kunnen zijn dat BNP naast preglomerulaire vaatverwijding ook voor post-glomerulaire vaatverwijding zorgt. De filtratiedruk in de nier zou op deze wijze hoog gehouden kunnen worden om meer bloed te filteren, zonder dat er een toename in doorbloeding optreedt.

In hoofdstuk 4 wordt de rol van intrarenaal endotheline-1 beschreven als mogelijke mediator van de effecten van BNP op de nier bij gezonden. Endotheline-1 zorgt namelijk naast vaatverwijding ook voor zoutuitscheiding door de nier. Dit effect komt tot stand via de zogenaamde endotheline-1 B receptor. Uit ons onderzoek blijkt echter dat selectief endotheline-1 B receptor antagonisme op zich wel leidt tot een verminderde zoutuitscheiding, maar dat het geen effect heeft op de door BNP teeweergebrachte toename in zoutuitscheiding en glomerulaire filtratie. Een eventuele rol van endotheline-1 via de endotheline A receptor zal in de toekomst nog nader onderzocht moeten worden.
Het onderzoek uit hoofdstuk 5 betreft het lokale effect van BNP in de onderarm van gezonde proefpersonen. In deze studie wordt het effect van BNP vergeleken met het effect van ANP. Ten eerste tonen we aan dat de effecten van beide natriuretische peptiden reproduceerbaar zijn in de tijd. Vervolgens zien we dat BNP voor een dosis-afhankelijke vaatverwijding zorgt, maar een geringer vaatverwijdend vermogen heeft dan ANP. Omdat tot dan alleen bekend was dat BNP en ANP via dezelfde receptor hun biologisch effect uitoefenen, ontstaat het vermoeden dat meer dan één mechanisme betrokken is bij de werking van BNP.

De onderliggende werkingsmechanismen van BNP in de onderarm worden nader onderzocht in hoofdstuk 6. Hieruit blijkt dat naast activatie van de natriuretische peptide receptor A en daarmee opening van kalium-kanalen, ook stimulatie van stikstofoxide (NO) leidt tot vaatverwijding in de onderarm. Hierbij worden tevens cGMP en c-type natriuretisch peptide geproduceerd.

In hoofdstuk 7 worden de resultaten van bovengeschreven onderzoeken besproken in de context van de huidige literatuur. Tenslotte wordt tevens de klinische relevantie aangegeven van de onderzoeksresultaten voor de behandeling en/of preventie van hoge bloeddruk en/of hartfalen.
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Dankwoord
Dankwoord

Het werken aan dit proefschrift was een leerzaam proces. Van een stagiair gezondheidswetenschappen gegroeid naar een zelfstandige onderzoeker. Een van de dingen die ik heb geleerd is, dat je je eigen mogelijkheden moet creëren om je doelstellingen te kunnen bereiken. Actief participeren in je omgeving met creativiteit, flexibiliteit en doorzettingsvermogen. Niet alleen wetenschappelijk, maar dus zeer zeker ook persoonlijk heb ik mij de afgelopen periode kunnen ontwikkelen, mede dankzij de bijdrage van de hier genoemde personen.

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