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# UCC

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**The Virtually Mature BNP (BNP1-32) is a Precursor for the More Effective BNP1-30**

**Short Title:** BNP1-30 is more effective than BNP1-32

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## **Abstract**

**Background and Purpose:** The B-type natriuretic peptide (BNP1-32) exerts vasorelaxing and cardioprotective activity. BNP is used as a biomarker for the diagnosis of cardiopathological conditions and recombinant BNP1-32 as a drug for the treatment of such. BNP1-32 has a short half-life time and thus, similar to other vasoactive peptides like angiotensin II and bradykinin, can be enzymatically truncated forming bioactive metabolites.

We aimed to investigate the metabolism of BNP1-32 in mouse lung, to identify potential new BNP metabolites and to disclose their biological activity compared to the BNP1-32, *in vitro* and *in vivo*.

**Experimental Approach:** Using High Performance Liquid Chromatography and Mass-Spectrometry, we identified a new BNP metabolite, BNP1-30, in the lung being generated by endothelin-converting enzyme-1.

**Key Results:** BNP1-30 is more efficient in stimulating the guanylyl cyclase receptor A (GC-A) and, in contrast to BNP1-32, is also able to profoundly stimulate the GC-B. *In vivo*, BNP1-30 reduced the mean arterial blood pressure of normotensive mice after acute infusion significantly more than BNP1-32. In a model of severe hypertension, a 3-day infusion of BNP1-30 was able to reduce systolic blood pressure by 30 mmHg and to improve markers of heart failure, while BNP1-32 was without significant effect.

**Conclusion and Implications:** Our results suggest that BNP1-32 is the precursor for the biologically more active BNP1-30 leading to a fundamental extension of the natriuretic-

peptide system. Due to expanded activity, BNP1-30 might be a promising treatment option for cardiovascular diseases. Furthermore, its potency as a new diagnostic marker of specific cardiac diseases should be evaluated.

**Keywords:** Cardiovascular Disease/Hypertension/Pharmacology/Natriuretic peptides/Peptide metabolism

### **Abbreviations**

BNP1-32 = B-type natriuretic peptide 1-32; BNP1-30 = B-type natriuretic peptide with 30 amino acids instead of 32 like in the original BNP1-32; CP = Carboxypeptidase; ECE-1 = Endothelin-converting enzyme-1; GC = guanylyl cyclase receptor; HDMEC = Human dermal microvascular endothelial cells; HEK293 cells = human embryonal kidney cells; HPLC = High Performance Liquid Chromatography, MALDI-TOF-MS = Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; MC = mesangial cells; NEP = Neprilysin; VSMC = Vascular smooth muscle cells, ZPP = Z-pro-prolinal

### **Bullet point summary**

What is already known:

- BNP1-32 is not the only cardiovascular protective BNP.

What this study adds:

- BNP1-32 is not the most active BNP peptide in the system, BNP1-30 is more effective.

Clinical significance:

- BNP1-30 could be a potential new treatment option or biomarker for specific cardiovascular diseases.
- Natriuretic peptide system needs to be revised and expanded.

## Introduction

Natriuretic peptides are naturally occurring cyclic hormones maintaining natriuresis and diuresis leading to reduced blood pressure and vascular volume (Potter et al., 2009). All natriuretic peptides share a highly conserved 17-amino-acid ring structure formed by a disulphide bridge (C<sup>10</sup>-C<sup>26</sup>). This intact ring system is the prerequisite for biological activity (Misono et al., 1984). The biological actions of natriuretic peptides are modulated by three different membrane-bound receptor subtypes, guanylyl cyclase receptor A, B, and C (GC-A, GC-B, and GC-C) (Potter et al., 2006). A-type natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are ligands of GC-A, while C-type natriuretic peptide (CNP) stimulates the GC-B. Both receptors contain an intra-cellular guanylyl cyclase domain, which can convert GTP to the second messenger cGMP after stimulation with its native ligands. GC-C has similar affinity to all three natriuretic peptides and is commonly considered a clearance receptor and is discussed to be involved in cardiovascular diseases (Rubattu et al., 2010).

BNP has gained increased importance as a biomarker in the clinical diagnosis of cardiovascular diseases, for risk stratification and guidance of therapy, since its plasma levels are elevated under such conditions and rise with the progression of the disease (Logeart, 2010, Korenstein et al., 2007). Moreover, Nesiritide®, the recombinant manufactured mature human BNP1-32, has been studied in many clinical trials and is discussed as a drug for the intravenous treatment of selected patients with acutely decompensated congestive heart failure (O'Connor et al., 2011, Pleister et al., 2011).

Catabolism of BNP was mainly regarded to be performed by neprilysin (E.C.3.4.24.11), but previously, we showed that all three natriuretic peptides have different degradation rates,

although they are structurally similar (Walther et al., 2004, Pankow et al., 2009). Compared to ANP and CNP, the degradation of BNP is virtually not affected by neprilysin. Instead, we identified BNP to be metabolised by meprin A in murine kidney into the N-terminal truncated and less biologically active metabolite BNP7-32 (Pankow et al., 2007).

## **Materials and Methods**

All human and mouse recombinant BNP metabolites were synthesised by Biosyntan (Berlin, Germany). The cGMP ELISA kit for cells or for plasma was purchased from Enzo Life Sciences (Loerrach, Germany). Recombinant Endothelin-converting enzyme-1 was purchased from R&D Systems (Wiesbaden, Germany). PolyFect® reagent was obtained from Qiagen GmbH (Hilden, Germany). GC-A was purchased from Origene Technologies (Rockville, Md). GC-B was provided as a gift by Michael Bader (MDC, Berlin, Germany). Cell culture products were purchased from Invitrogen (Karlsruhe, Germany). The QuantiTect® Reverse Transcription Kit and the QuantiTect® SYBR® Green PCR Kit were used from Qiagen GmbH. All other chemicals used were obtained from Sigma (Taufkirchen, Germany). Animals were purchased from Charles River® (Sulzfeld, Germany and Margate, UK).

## **Human and mouse lung preparation**

Human (healthy tissue provided by the University Hospital Giessen) and mouse (C57B/L6J [RRID:MGI:5657312]) lung homogenates were prepared by homogenising lung tissue in a 50 mM Tris buffer (pH 7.4) with a homogeniser. To generate mouse lung membranes, homogenised samples were centrifuged at 40,000 g to separate membranes from the cytosol. After a washing step, the pellet was resuspended in 50 mM Tris buffer and stored at -80°C.

The total protein content of the membrane preparations was determined by BCA assay and adjusted depending on the experiment.

#### **Degradation of mBNP1-32 by mouse lung membrane preparations**

Ten  $\mu\text{M}$  of mBNP1-32 was incubated with murine lung membrane preparations (60  $\mu\text{g}$  protein 100  $\mu\text{M}$  buffer) in Tris buffer (50 mM, pH 7.5) supplemented with 0.1% BSA (Tris/BSA - buffer) at 37°C under constant shaking at 300 U min<sup>-1</sup>. The buffer was supplemented with traces of BSA to minimise adhesion of the peptides on the tube walls. Reactions were stopped after 0, 20, 40, 60, and 80 min by a pH change with perchloric acid. After centrifugation, supernatants were analysed by Liquid Chromatography Mass Spectrometry (LCMS) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry.

#### **Degradation of hBNP by human lung preparations**

Ten  $\mu\text{M}$  of hBNP1-32 or hBNP1-30 were incubated with human lung homogenate (90  $\mu\text{g}$  protein 150  $\mu\text{L}$  buffer) in Tris buffer (50 mM, pH 7.5) with 0.1% BSA (Tris/BSA - buffer) at 37°C under constant shaking at 300 U min<sup>-1</sup>. The reactions were stopped after 0 and 10 min by addition of perchloric acid. The samples were then centrifuged and the supernatants were analysed by LCMS. For comparison of mBNP1-30 and hBNP1-30 degradation, equal concentrations of mouse and human homogenate were used (90  $\mu\text{g}$  protein 150  $\mu\text{L}$  buffer), respectively.

### **Generation of mBNP1-30 with lung membrane preparations in the presence of enzyme inhibitors**

Ten  $\mu\text{M}$  of mBNP1-32 were incubated with 60  $\mu\text{g/mL}$  protein of organ membrane preparations in Tris/BSA buffer (50 mM pH 7.5) in the presence of the following enzyme inhibitors: actinonin against meprin A [10  $\mu\text{M}$ ], captopril against Angiotensin-converting enzyme [10  $\mu\text{M}$ ], Carboxypeptidase inhibitor [10  $\mu\text{M}$ ], Chymostatin against serine and cysteine peptidases [5  $\mu\text{M}$ ], EDTA against metallopeptidases [5  $\mu\text{M}$ ], F480 against Angiotensin-converting enzyme-2 [1  $\mu\text{M}$ ], Leupeptin against serine and cysteine peptidases [10  $\mu\text{M}$ ], pepstatin against aspartylpeptidases [10  $\mu\text{M}$ ], Roche cocktail inhibitor 10x [1  $\mu\text{L}$ ], SM19712 against endothelin-converting enzyme 1 [10  $\mu\text{M}$ ], trypsin inhibitor against trypsin-like peptidases [10  $\mu\text{M}$ ], Z-pro-prolinal against prolylcarboxypeptidases [100  $\mu\text{M}$ ] in a reaction volume of 100  $\mu\text{L}$ . Samples were incubated at 37°C under constant shaking at 300 U  $\text{min}^{-1}$ . Reactions were stopped after 60 min by inducing a pH change by supplementation of perchloric acid. After centrifugation supernatants were analysed by liquid chromatography mass spectrometry and Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry.

### **Degradation of natriuretic peptides by recombinant Endothelin-converting enzyme-1**

Murine ANP, BNP1-32, and CNP were incubated with 0.4 mU of recombinant Endothelin-converting enzyme-1 in Tris/BSA buffer (50 mM, pH 7.5). Reactions were stopped after 0, 20, 40, and 60 min by a pH shift with perchloric acid. After centrifugation, supernatants were analysed by liquid chromatography mass spectrometry.



## **Liquid Chromatography Mass Spectrometry (LCMS)**

Murine peptide analysis was performed by reversed phase chromatography using an LCMS with UV-detection from Shimadzu (Duisburg, Germany). Each murine sample was loaded onto a RP Nucleosil 100 C12 column (250 x 4.6 mm, 5  $\mu\text{m}$ ) from Phenomenex (Aschaffenburg, Germany) with a flow rate of 1  $\text{mL min}^{-1}$ . Human samples were analysed after being injected onto a RP Jupiter Proteo C12 column (250 x 4.6 mm, 4  $\mu\text{m}$ ) from Phenomenex with a flow rate of 0.5  $\text{mL min}^{-1}$ . The peptides were detected at 206 nm and a linear correlation between peak areas and natriuretic peptide concentrations was confirmed before analysis. BNP1-32 and its degradation products were eluted within 35 min using a linear gradient of acetonitrile and ultrapurified water laced with 0.05% trifluoroacetic acid for murine samples or 0.10% formic acid for human samples. After separation, peaks were manually fractionated for the analysis with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry.

## **Matrix-Assisted Laser Desorption/ Ionisation Time-of-flight Mass Spectrometry (MALDI-TOF MS)**

MALDI-TOF-MS was performed on an Ultraflex I TOF/TOF mass spectrometer equipped with a nitrogen laser and a LIFT-MS/MS facility from Bruker Daltonics (Bremen, Germany). The instrument was operated in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid and methylendiphosphonic acid as matrix. Sum spectra consisting of 200–400 single spectra were acquired. 1  $\mu\text{L}$  of the dried sample was mixed with 1  $\mu\text{L}$  of the matrix dissolved in trifluoroacetic acid in acetonitrile-water and placed on the MALDI plate, followed by spot analyses. For external calibration a peptide standard from Bruker Daltonics) was used. For data processing and instrument control, the Compass 1.1 software package consisting of FlexControl 2.4, FlexAnalysis 3.0, and BioTools 3.0 was used.

## Cell Culture and transfections

Human embryonic kidney cells (HEK293 [RRID:CVCL\_0045]) were cultured in DMEM supplemented with 10% FBS and penicillin–streptomycin ( $100 \text{ IU mL}^{-1}$  to  $100 \mu\text{g mL}^{-1}$ ). The cultures were maintained at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified incubator. One day after seeding, HEK293 cells were transferred into 24-well cell culture dishes. Transfections with plasmids encoding murine GC-A or GC-B were performed using PolyFect<sup>®</sup> reagent in DMEM with 0.5% FBS. Cells transfected with the empty plasmid served as controls. After 24 hours, cells were used for bioactivity measurements.

Human dermal microvascular endothelial cells (Zhu et al., 2013) were cultured in Endothelial Cell Basal Medium supplemented with growth factors present in the Endothelial Cell Supplement Kit from PromoCell. Vascular smooth muscle cells and mesangial cells were isolated from the explants of the thoracic aortas (Zhu et al., 2013) or kidneys (Tetzner et al., 2016) of 4 to 6-week-old C57BL/6 animals. Vascular Smooth muscle cells were grown in DMEM supplemented with 10% FBS,  $100 \text{ IU mL}^{-1}$  penicillin, and  $100 \mu\text{g mL}^{-1}$  streptomycin as described previously (Pankow et al., 2007). Kidneys were decapsulated and minced in ice-cold RPMI medium. Homogenates were dispersed with 5 mL chilled RPMI medium to a  $100 \mu\text{m}$  nylon filter and then the filtrate transferred to a  $70 \mu\text{m}$  nylon filter. After decapsulated, the glomerular ‘cores’ were digested with collagenase type II for approximately 30 min, then centrifuged at 1000 rpm for 10 min. The pellet was resuspended in RPMI 1640 medium supplemented with 17% FBS, penicillin/streptomycin, and  $0.1 \text{ U mL}^{-1}$  insulin. The cultures were placed at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator (Tetzner et al., 2016).

## Bioactivity of natriuretic peptides *in vitro*:

Transfected HEK293 cells, human dermal microvascular endothelial cells, vascular smooth muscle cells, and mesangial cells were treated with vehicle (Tris buffer), or vehicle

containing 1  $\mu$ M of the respective natriuretic peptide for 5 min. After washing 3 times in cold Hank's balanced salt solution, cells were resuspended in 150  $\mu$ L of cell lyses buffer provided in a cGMP kit. Concentrations of cGMP were determined according to the instructions of the manufacturer and were measured at 405 nm with a correction at 570 nm and expressed as picomoles per millilitre as shown previously (Zhu et al., 2013, Pankow et al., 2007, Pankow et al., 2009). All experiments have an n of = 3 in triplicates.

### **Bioactivity measurement *in vivo***

All animals were maintained under standardized conditions with an artificial 12-hour dark/light cycle, with free access to food and water. Experiments on animals, blood collection, and organ explanations were performed in accordance with the Animals (Scientific Procedures) Act 1986 of the Parliament of the United Kingdom, the Federal Law on the Use of Experimental Animals in Germany, guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the local authorities (Home Office, London, UK; Regierungspraesidium Giessen, Germany). Human tissue samples were obtained and handled in adherence to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001.

### **Bioactivity and hemodynamic measurements in mice**

Male C57B/L6J mice (16 weeks, n=26, vehicle arm n=5, mBNP1-30, mBNP1-32, and mBNP1-45 arms n=7) were randomised into groups, whereby the experimenter was blinded to treatment. Mice were anaesthetised with 4% isoflurane and intubated for artificial ventilation with a mixture of 20% oxygen and 80% room air through a rodent ventilator, to

which 2% to 2.5% isoflurane was added for continuous anaesthesia. During surgery, animals were kept on a homoeothermic blanket to maintain body temperature. The right common carotid artery and the right internal jugular vein were dissected. A Mikro-Tip<sup>®</sup> mouse pressure catheter from Millar Instruments, Texas was inserted into the carotid artery and a cannula was inserted into the jugular vein. Baseline blood pressure and heart rate were monitored and recorded for 10 minutes with a pressure transducer system and a blood pressure monitoring software from TSE systems GmbH, Germany. Through the cannula, a single bolus injection of murine BNP1-32, BNP1-30, BNP1-45 (100  $\mu\text{L}$  30g<sup>-1</sup> body weight of a  $5 \times 10^{-6}$  M solution [1.67 nmol peptide g<sup>-1</sup> body weight]), or saline was injected. The hemodynamic parameters were measured for 4 min after injection. Subsequent to the measurement, EDTA blood was taken by puncturing of the left ventricle. Blood samples were centrifuged, and the plasma was snap frozen in liquid nitrogen and stored at -80°C until further processing. Plasma cGMP levels were measured after an extraction process using 95% ethanol by the cGMP complete ELISA Kit for plasma samples according to the manufacturer's instructions.

### **Bioactivity and hemodynamic measurements in spontaneously hypertensive rats (SHR)**

Osmotic minipumps from Alzet, California (pump rate: 0.5  $\mu\text{L hr}^{-1}$ ) were put into sterile saline solution for three hours for preconditioning. After that, minipumps were filled each with either sterile saline, or filtered solutions of BNP1-32 or BNP1-30 to allow the administration of 300 ng kg<sup>-1</sup> min<sup>-1</sup>. Male spontaneously hypertensive rats (RRID:RGD\_61000) at the age of 12 weeks (n=18, 6 mice per arm; vehicle, mBNP1-32, and mBNP1-30) were randomised into groups, whereby the experimenter was blinded to treatment. Rats were anaesthetised through injection with ketamin/xylocaine/saline (3:0.5:6.5; 100 mm<sup>3</sup> 100g<sup>-1</sup>) and put under an infrared heating lamp to maintain body temperature. A

small part of the fur on the backside was removed using depilatory cream. Next, a primed osmotic minipump was implanted under the skin on the backside of the animal. Wounds were closed with clips. After the animals woke up, they were kept in appropriate cages with normal housing and food for three days. For the determination of hemodynamic parameters, animals were anesthetised with 4% isoflurane and were kept ventilated with a mixture of 20% oxygen and 80% room air through a rodent ventilator, to which 2% to 2.5% isoflurane was added for continuous anaesthesia. Animals were kept on a homoeothermic blanket to maintain body temperature. The right common carotid artery was dissected. A Mikro-Tip<sup>®</sup> rat pressure catheter from Millar Instruments, Texas was placed into the right carotid artery to measure arterial blood pressure and heart rate. Parameters were recorded for 10 minutes with a blood pressure monitoring software from TSE systems GmbH, Germany. Finally, EDTA blood was taken by puncturing of the left ventricle. Blood samples were centrifuged, and the plasma was snap frozen in liquid nitrogen and stored at 193.15K until further processing. Plasma cGMP levels were measured using the cGMP complete ELISA Kit for plasma samples according to the manufacturer's instructions.

### **Real time PCR**

The quantitative real-time polymerase chain reaction (RT-PCR) was carried out in three steps including isolation of RNA, reverse transcription, and SYBR<sup>®</sup> Green-based RT-PCR. After cardiac RNA isolation, 1 µg of RNA was used to transcribe cDNA with the QuantiTect<sup>®</sup> Reverse Transcription Kit from Qiagen according to the manufacturer's instructions. Quantitative real-time PCR was carried out using specific primers for rat ANP and BNP with the QuantiTect<sup>®</sup> SYBR<sup>®</sup> Green PCR Kit from Qiagen. Melting curve analyses were performed to monitor PCR product purity. To compare the different treatment groups to the saline treated rats, data was expressed as relative values (changes).

## Statistics

For statistical comparisons, Student's *t* test or One-way ANOVA with Bonferroni's Multiple Comparison (Graph Pad Prism 5.01 [RRID:SCR\_002798]; Graph Pad Software Inc., San Diego, California, USA) were used (means  $\pm$  SEM). Two-way ANOVA was used to compare BNP1-32 and BNP1-30 in dose-response curves. Post-hoc tests were only performed if *F* was significant and there was no variance inhomogeneity. EC<sub>50</sub> values were calculated by using sigmoidal dose response curve fit in (Graph Pad Prism 5.01; Graph Pad Software Inc., San Diego, California, USA). Significance was considered at a value of *P* < 0.05.

## Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18: G protein- coupled receptors (Alexander et al., 2017).

## Results and Discussion

Besides kidney, other organs have a high metabolic activity towards natriuretic peptides. Our investigation of the initial metabolism of BNP1-32 in such organs revealed the occurrence of another BNP metabolite, generated by an enzyme from murine lung. As seen in **Figure 1A**, BNP1-32 was degraded by lung membrane preparations over time resulting in the generation of an initial and dominant cleavage product (**Fig. 1B**; retention time 12.34 min). This product was analysed qualitatively by LCMS and moreover fractionated after LC separation for additional analysis by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. With both methods a mass of approximately 3,329 Daltons was found which

could be assigned to a C-terminal truncated 30 amino acid long analogue of BNP (**Fig. 1C**), named BNP1-30. Other than in our former investigations in kidney, we did not find a peak of BNP7-32 in the chromatograms even upon enhanced incubation times, suggesting that this metabolite was not produced in lung.

With the help of enzyme inhibitors, we aimed to discover the peptidase responsible for BNP1-30 generation. **Figure 2A** shows the amount of BNP1-30 accumulated through incubation with membrane preparations of the lung in the presence of enzyme family-specific inhibitors (white bars) or enzyme-specific inhibitors (grey bars). The amount of BNP1-30 generated with the membranes in the absence of peptidase inhibitors was set to 100% (black bar). EDTA, a metallopeptidase inhibitor, inhibited the generation of BNP1-30 by approximately 50% while other enzyme-family specific inhibitors had no significant influence. Therefore, a collection of specific metallopeptidase inhibitors were used and it was found that the specific inhibitor for the endothelin-converting enzyme 1, SM-19712, was effective in inhibiting BNP1-30 generation. Consequently, generation of BNP1-30 by lung membranes was compared over time in the presence or absence of a specific Endothelin-converting enzyme-1 inhibitor (**Fig. 2B**). Inhibition of the peptidase clearly reduced accumulation of BNP1-30 over time. Subsequently, recombinant Endothelin-converting enzyme-1 was incubated with BNP1-32 to investigate whether the peptidase is able to generate BNP1-30. As seen in **Figure 2C** by the overlay of the chromatograms generated from incubation of BNP1-32 with lung membrane preparations or with recombinant endothelin-converting enzyme-1, a cleavage product with the same retention time was generated. For additional proof, the cleavage product from endothelin-converting enzyme-1 incubation was fractioned and analysed with matrix-assisted laser desorption/ionisation time-

of-flight mass spectrometry which revealed that the peak indeed represents the metabolite BNP1-30.

To disclose a possible impact of endothelin-converting enzyme-1 on other natriuretic peptides, the peptidase was incubated with murine ANP and CNP and its catabolism compared towards that of mBNP1-32. The peptidase was not able to cleave the other two natriuretic peptides (**Fig. 2D**).

As seen in **Figure 1C**, BNP1-30 still provides the disulphide-linked loop structure, which is essential for biological activity of natriuretic peptides (Potter et al., 2009). Thus, we hypothesised that BNP1-30 is still bioactive and tested its potency to stimulate GC-A and GC-B in transfected HEK293 cells by quantifying the formation of the second messenger cGMP. As previously shown (Potter, 2011, Pankow et al., 2007), ANP and BNP stimulated their native receptor GC-A, but could not profoundly stimulate the cGMP generation via GC-B. The same result is seen in **Figure 3A&B**. The new BNP metabolite BNP1-30 was also able to stimulate GC-A (**Fig. 3A**). Interestingly, the formation of cGMP was significantly higher in BNP1-30-stimulated cells compared to BNP1-32-stimulated cells indicating that BNP1-30 is more bioactive on the GC-A compared to BNP1-32. This is different to the previously identified metabolite BNP7-32 whose ability to stimulate the receptor was only half of that compared to BNP1-32 (Pankow et al., 2007). Although ANP and BNP1-32 could only marginally stimulate the GC-B, BNP1-30 showed a much more pronounced effect on GC-B transfected cells, which was still low, compared to its effect on GC-A, but was 3-fold higher in comparison to BNP1-32. Of note, none of the peptides could increase cGMP levels in control vector-transfected cells (data not shown).



To test whether the cGMP generation stimulated by either GC-A or GC-B can be additive, double-transfected HEK293 cells were used. Dose-response curves identified BNP1-30 to be more efficient. A lower half maximal effective concentration ( $EC_{50}$ ) also indicated a higher potency compared to BNP1-32, but this difference was not significant (**Fig. 3C**). Investigation of primary cells being essential in blood pressure and sodium homeostasis showed that stimulation of either primary endothelial cells (EC), dominantly expressing endogenous GC-A, or mesangial cells, primarily expressing endogenous GC-B, with BNP1-30 was significantly more efficient compared to stimulation with BNP1-32 (**Fig. 3D&E**) (Zhu et al., 2013). Similar results were observed in vascular smooth muscle cells, expressing both receptors (**Fig. 3F**) (Zhu et al., 2013).

To evaluate whether the higher efficiency of the new BNP metabolite in a variety of different primary cell types correlates to *in vivo* actions, we first investigated the acute biological effect of BNP1-32 and BNP1-30 on blood pressure in a normotensive animal model. In a first experiment, each peptide or saline as a control was injected into the jugular vein of wild-type mice after ensuring stable baseline blood pressure conditions. Infusion of either BNP1-32 or BNP1-30 resulted in a transient decrease in blood pressure compared to saline injection (**Fig. 4A**). However, the new metabolite BNP1-30 caused a significant higher decrease in the mean arterial blood pressure that was almost doubled compared to that of the mature BNP1-32. Notably, the time to reach its maximal vasorelaxant effect was not different between both peptides (BNP1-32:  $3.8 \pm 0.7$  min; BNP1-30:  $3.3 \pm 1.2$  min). This physiological effect was associated with a more pronounced increase in cGMP levels in the plasma of the BNP1-30-treated animals compared to the BNP1-32 treated animals (**Fig. 4B**), strongly supporting our *in vitro* data (**Fig. 3**). In an independent set of animals, BNP1-30 was compared to BNP1-45, an alternatively spliced BNP metabolite. This molecule was found only in rodents and has

been discussed to be the active BNP fragment in the murine system (Ferdinal et al., 2009, Steinhilper, 1993, Nakagawa et al., 1995). Although, again both peptide infusions caused a significant decrease in blood pressure compared to the saline infusion, the effect of BNP1-30 was almost doubled in comparison to BNP1-45 (**Fig. 4C**).

Additionally, BNP1-32 and BNP1-30 were tested in a hypertensive animal model to compare their blood-pressure lowering effects under pathophysiological conditions. In contrast to the experiments in mice, the peptides or saline were administered through an osmotic minipump into 3-month old SHR for three days to evaluate longer-lasting blood pressure effects. Hemodynamic measurement at the end of the 3 days showed the excessive blood pressure elevation described for such SHR (Pacheco et al., 2011) with systolic blood pressure (SBP) at  $193.9 \pm 10.8$  mmHg in the control group receiving saline. While there was no significant effect of BNP1-32 ( $\Delta$ SBP:  $-7.6 \pm 4.4$  mmHg), the administration of BNP1-30 lowered the SBP by 16 % ( $\Delta$ SBP:  $-30.6 \pm 6.7$  mmHg) (**Fig. 5A**). Quantitative real time PCR revealed a significant reduction of cardiac ANP mRNA in both treatment groups, whereby this effect was more pronounced for BNP1-30 treated animals, most probably due to the reduced wall tension in the heart achieved through the reduction of blood pressure (**Fig. 5B**). Such effect was even more pronounced for BNP mRNA. Cardiac BNP mRNA levels in BNP1-30-treated animals were significantly reduced while BNP1-32 treatment was without effect (**Fig. 5C**). A possible negative feedback mechanism on BNP generation through the administration of BNP seems not to be present, since BNP levels are unaltered in BNP1-32-treated animals. Since BNP is used as a biomarker for the progression of cardiovascular diseases (Arakawa et al., 1994, Davis et al., 1994), the decrease of BNP mRNA through the treatment of BNP1-30 indicates an improvement of cardiovascular health of the animals. Obviously, this improvement was not achieved with the conventional BNP1-32-treatment.

These *in vivo* results identify BNP1-30, due to its potent actions on blood pressure, as a very promising therapy option for the treatment of hypertension. This direct hypotensive action might be further pronounced by additional stimulation of long-lasting natriuresis. Although the effects of BNP1-30 need to be further investigated in regards to this, its potent impact on both natriuretic peptide receptors and thus on blood pressure makes it likely to also exert at least the natriuretic properties of the 'mature' BNP1-32, which is already used in the therapy of congestive heart failure (O'Connor et al., 2011, Pleister et al., 2011). The actions of BNP1-30 are not only stronger compared to BNP1-32 but also more complex, since the beneficial effects of the GC-B signalling pathway are also given through the ability of BNP1-30 stimulate this receptor. Because of this extended receptor profile, BNP1-30 should be considered as a better treatment option in heart failure patients, where GC-A activity is significantly decreased and GC-B accounts for the majority of natriuretic peptide-dependent activity in the heart (Dickey et al., 2007). Thus, it should be soon evaluated from the clinical perspective whether the therapeutic benefits generated with Nesiritide<sup>®</sup> can be further improved by the use of BNP1-30.

However, it could be argued that the BNP1-30 has no clinical importance as it might not be present in a human body. While Niederkofler *et al.* could demonstrate the generation of a BNP1-30 peak when incubating hBNP1-32 with human heparin plasma samples (Niederkofler et al., 2008), we performed a set of experiments to identify BNP1-30 in the human lung. **Figs. 6A&B** show the retention times and associated mass spectra of recombinant human BNP1-32 and BNP1-30, respectively. While the full peak of human BNP1-32 was initially present after addition to human lung homogenate, incubation for 10 min led to a decreased peak area and to the occurrence of (**Fig. 6C**) another small peak, identified as human BNP1-30 (**Fig. 6D**) based on retention time and *m/z* values.

However, relative to the mouse, much less human BNP1-30 was detected using human lung material. To answer the question whether this result is based on less generation or more rapid degradation of human BNP1-30, we performed an additional experiment comparing the conversion rate of human and murine BNP1-30 in their species-respective lung material. After a 10 minute incubation, only  $19.6 \pm 1.6\%$  of the original human BNP1-30 remained; as opposed to the murine BNP1-30, which had  $83.9 \pm 7.5\%$  remaining. These results indicate that the less pronounced BNP1-30 peak in the human system is based on a faster degradation. This discovery further justifies the clinical relevance of this study by proving that BNP1-30 is present in human and is not limited to the mouse system.

BNP is an excellent marker to identify patients with cardiac pathology; however, it fails to discriminate between different cardiac diseases. It might be mentioned, that levels of BNP measured in plasma by commonly used kits (e.g. Triage<sup>®</sup>) do not consist of BNP1-32 only. The antibodies used in the routine measurements of BNP levels in cardiovascular and intensive care medicine cross-react with other molecular forms of BNP, since they detect epitopes within the ring structure of the peptides and thus can also detect shorter fragments (Korenstein et al., 2007, Nishikimi et al., 2011). Such antibodies can also not distinguish between shorter BNP metabolites still having intact 17-aa ring structures and thus bind to BNP1-32 as well as to its metabolites, including BNP1-30. At the moment, no antibodies are available to specifically detect BNP metabolites. Furthermore, the concentration of single BNP metabolites in the blood might be in the low picomolar or even femtomolar range and thus very sensitive methods for the detection and quantification of such small quantities of single BNP metabolites need to be established.

However, the generation of such specific antibodies that would allow the detection and quantification of defined BNP metabolites in plasma would lead to the generation of a BNP

metabolite profile for each patient enabling a completely new strategy in cardiovascular medicine. The determination of such a metabolite profile might allow the differentiation between diverse cardio-pathologies assuming that the etiopathology specifically changes the composition of the BNP pool due to differential regulations on the e.g. peptidase level leading to altered availability of BNP metabolising enzymes causing a disease-specific metabolite fingerprint.

## **Conclusion**

Our results suggest that BNP1-32 functions as the precursor for the more biologically active BNP1-30. The manipulation of the length of the C-terminus of BNP changes the receptor profile, as the endogenous BNP1-30 is, additionally to a significantly stronger stimulation of the GC-A compared to BNP1-32, also able to stimulate the second natriuretic peptide receptor, GC-B. The stronger ability of stimulating GC-A makes BNP1-30 in general a more effective treatment option of cardiovascular disease conditions like hypertension. Additionally, its receptor profile also enables a better treatment for heart failure patients. In these patients the main BNP1-32 receptor, GC-A, is dramatically downregulated which weakened the effectiveness of nesiritide, the recombinant form of BNP1-32, used as a drug for the treatment of decompensated heart failure in the clinics. As a result of the higher efficacy and unique receptor profile, BNP1-30 is certainly the better treatment option under such conditions and a very promising compound for the treatment of a broad variety of life-threatening cardiovascular diseases. The more effective stimulation opens up completely new perspectives for the design of natriuretic peptide agonists to treat a broad range of cardiovascular diseases.

### **Author Contributions**

A.S., Y.W., and X.Z. performed the majority of the laboratory experiments. A.M. provided follow-up experiments and language/formatting review. K.P. and W.E.S. performed part of the analytical experiments. T.W. designed the study, supervised the research, and oversaw statistics and the editing of the manuscript.

### **Competing Interests' Statement**

None

### **Declaration of transparency and scientific rigour**

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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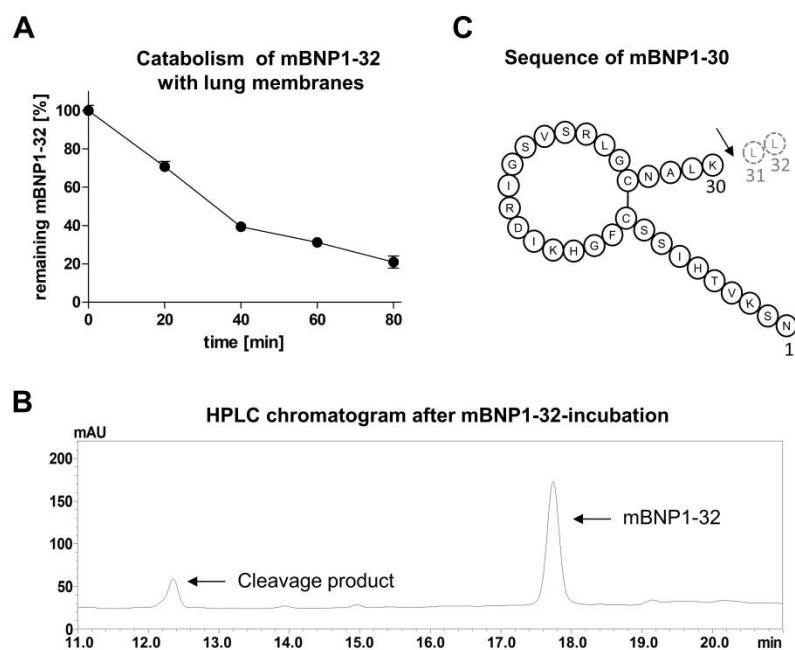
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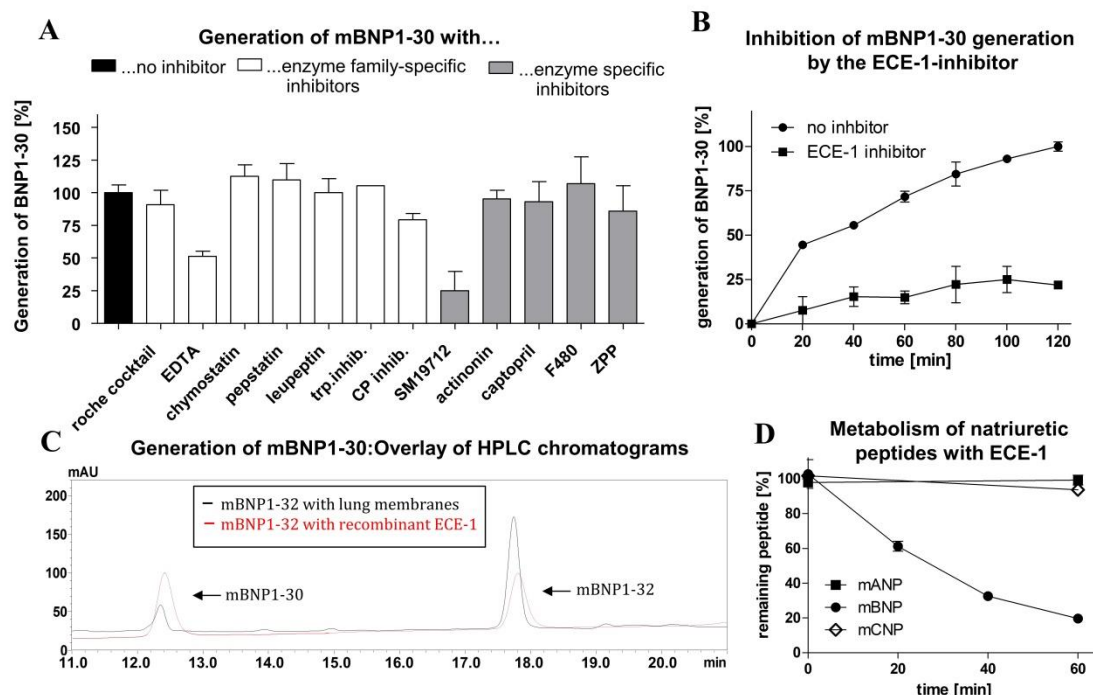
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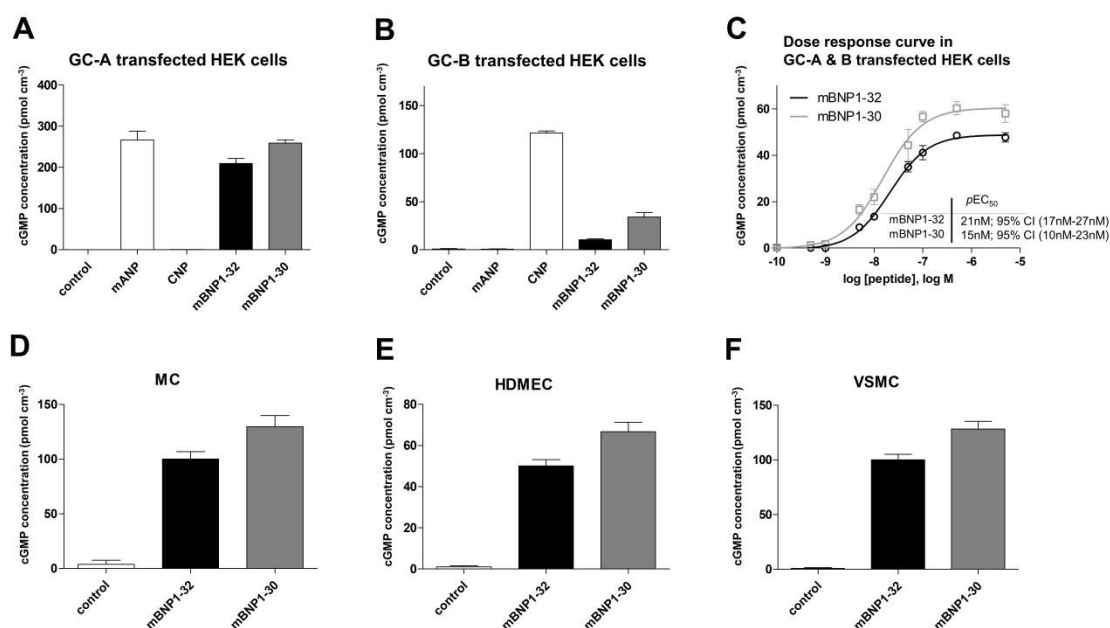
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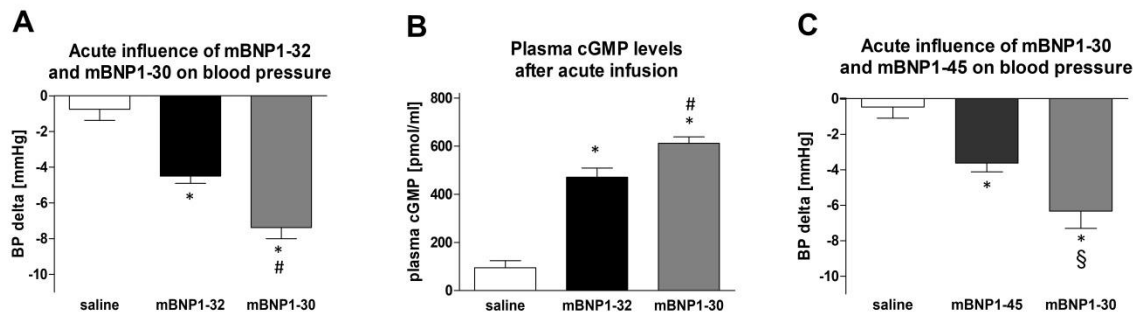
**Figure 1: Identification of a new mBNP metabolite:** **A)** Degradation of mBNP1-32 with murine lung membrane preparations over time (n = 3). Remaining concentration of mBNP1-32 in percent; **B)** Representative HPLC chromatogram of mBNP1-32 and product peak after 20 min incubation with murine lung membrane preparations; **C)** conducted secondary structure of mBNP1-30 and indication of cleavage site.



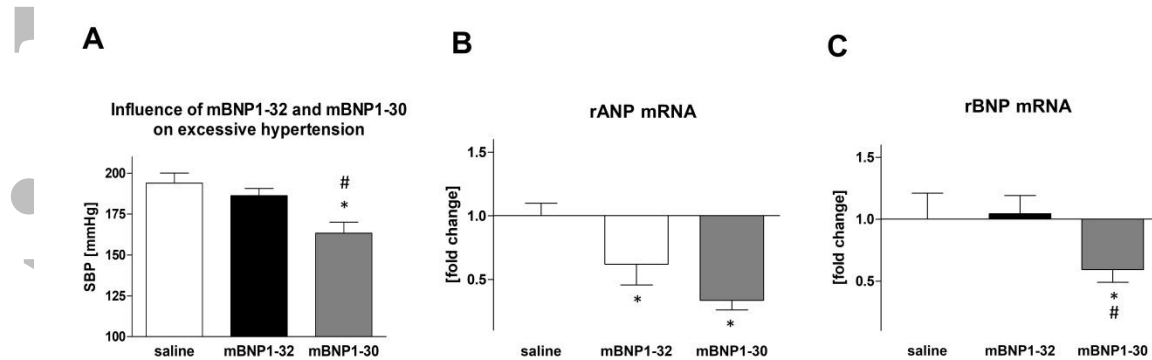
**Figure 2: Investigation of the peptidase responsible for the generation of the new metabolite mBNP1-30:** **A)** mBNP1-30 generation within 60 min through enzyme activity in lung membrane preparations with or without the presence of enzyme inhibitors. Generation of mBNP1-30 without inhibitors was set to 100% (black bar); **B)** Generation of mBNP1-30 using lung membrane preparations with and without the specific ECE-1 inhibitor SM19712 over time; generation of mBNP1-30 at 120 min was set to 100%; **C)** overlay of HPLC chromatograms of BNP1-32 and product peak (BNP1-30) after incubation of BNP1-32 with recombinant ECE-1 (in red) or after incubation with murine lung membrane preparations (in black). **D)** Metabolism of murine ANP, BNP, and CNP by recombinant ECE-1 over time; Where error bars are not indicated, SEM is within data symbols. (n = 3 in triplicates). Abbreviations: trp.inhib. = trypsin inhibitor, CP inhib. = carboxypeptidase inhibitor, mANP = murine atrial natriuretic peptide, mCNP = murine C-type natriuretic peptide.



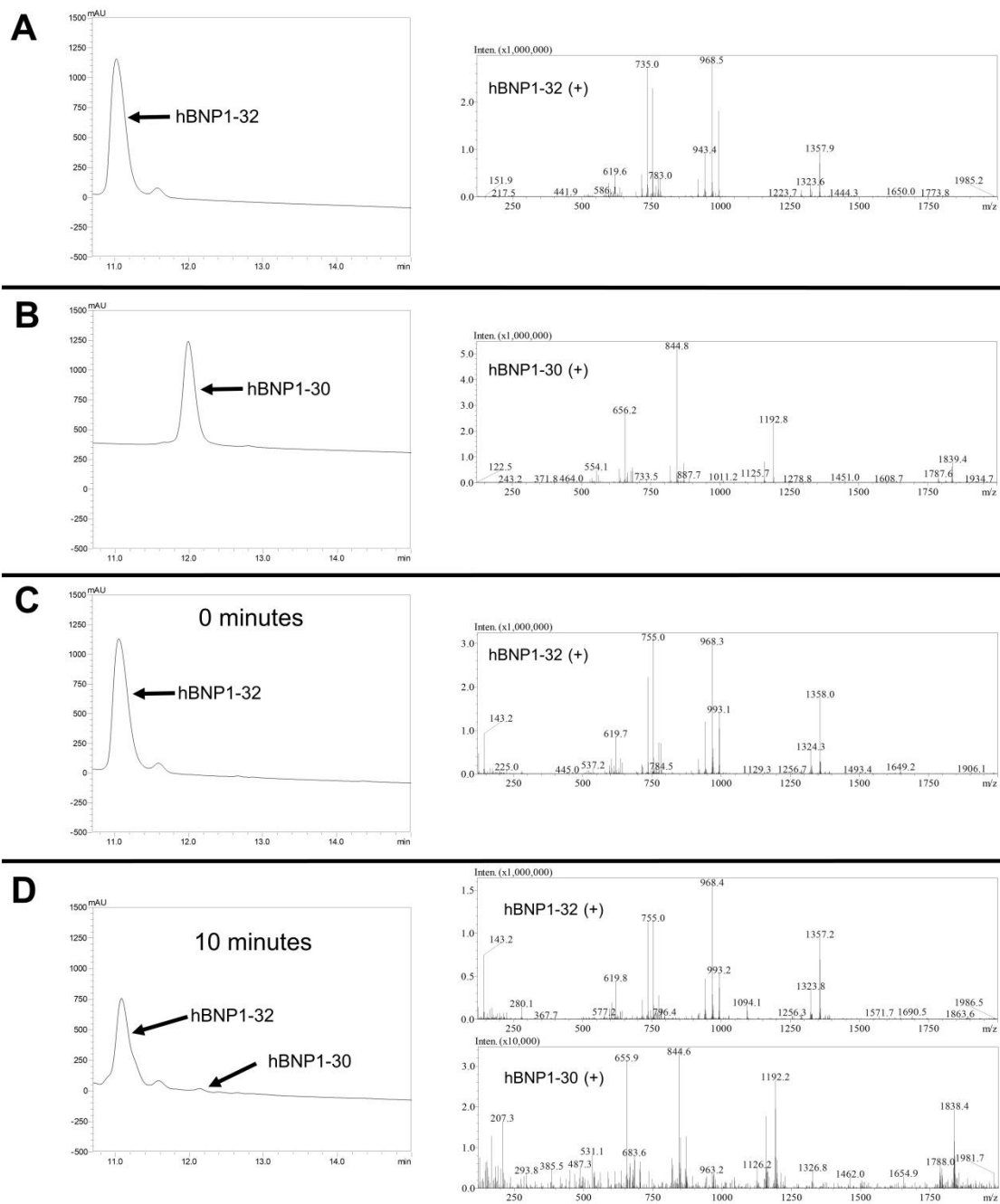
**Figure 3: Comparison of natriuretic peptides' bioactivity in vitro:** **A)** cGMP levels [pmol mL<sup>-1</sup>] after stimulation of GC-A-transfected HEK cells using 10<sup>-6</sup> M of the peptides; **B)** cGMP levels [pmol mL<sup>-1</sup>] after stimulation of GC-B-transfected HEK cells with 10<sup>-6</sup> M of the peptides; **C)** dose-response-curve of stimulated GC-A & GC-B double-transfected HEK cells; **D)** levels of cGMP [pmol mL<sup>-1</sup>] after stimulation of MC; **E)** levels of cGMP [pmol mL<sup>-1</sup>] after stimulation of human dermal microvascular endothelial cells; **F)** levels of cGMP [in percent] after stimulation of primary VSMC (D-F: peptide concentration of 10<sup>-6</sup> M); Where error bars are not indicated, SEM is within data symbols. (n = 3 in triplicates). Abbreviations: mANP= murine atrial natriuretic peptide, mBNP= murine B-type natriuretic peptide, mCNP= C-type natriuretic peptide.



**Figure 4: Acute influence of mBNP peptides on blood pressure in normotensive animal models:** **A)** Blood pressure delta [mmHg] after bolus injection of mBNP1-32 or mBNP1-30 ( $100 \mu\text{L } 30\text{g}^{-1}$  body weight of a  $5 \times 10^{-6}$  M solution [ $1.67 \text{ nmol peptide g}^{-1}$  body weight]) in comparison to saline in an acute phase experiment in a normotensive mouse model; **B)** cGMP levels [ $\text{pmol cm}^{-3}$ ] in plasma samples obtained from mBNP1-32-, mBNP1-30- and saline-injected animals; **C)** Acute blood pressure changes [mmHg] in an independent set of normotensive mice using mBNP1-45, mBNP1-30, or saline. ( $n = 7$  per peptide,  $n = 5$  for saline) (\*  $P < 0.05$  vs. control; #  $P < 0.05$  vs. BNP1-32; §  $P < 0.05$  vs. BNP1-45). Abbreviations: BP=Blood pressure.



**Figure 5: Long-term influence of BNP peptides on the blood pressure in a hypertensive animal model:** **A)** Change in systolic blood pressure [mmHg] in spontaneously hypertensive rats after continuous administration of mBNP1-32, mBNP1-30, or saline for three days; **B)** Cardiac ANP mRNA levels, and **C)** Cardiac BNP mRNA levels in fold change in the spontaneously hypertensive rats after administration of mBNP1-32, mBNP1-30 or saline for three days. (n = 6 per peptide or saline) (\*  $P < 0.05$  vs. control; #  $P < 0.05$  vs. BNP1-32). Abbreviations: SBP=Systolic blood pressure, mANP=mouse atrial natriuretic peptide.



**Figure 6: Generation of hBNP1-30 from hBNP1-32 in human lung homogenate: A)** Representative HPLC chromatogram and mass spectrum of pure hBNP1-32 peptide. **B)** HPLC chromatogram and mass spectrum of pure hBNP1-30 peptide. **C)** HPLC chromatogram and mass spectrum after 0 minutes of incubation with hBNP1-32. **D)** HPLC chromatogram and mass spectra after a 10-minute incubation with hBNP1-32.