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Attenuation of Monocyte Chemotaxis—A Novel Anti-inflammatory Mechanism of Action for the Cardio-protective Hormone B-Type Natriuretic Peptide

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Abstract B-type natriuretic peptide (BNP) is a prognostic and diagnostic marker for heart failure (HF). An anti-inflammatory, cardio-protective role for BNP was proposed. In cardiovascular diseases including pressure overload-induced HF, perivascular inflammation and cardiac fibrosis are, in part, mediated by monocyte chemoattractant protein (MCP)1-driven monocyte migration. We aimed to determine the role of BNP in monocyte motility to MCP1. A functional BNP receptor, natriuretic peptide receptor-A (NPRA) was identified in human monocytes. BNP treatment inhibited MCP1-induced THP1 (monocytic leukemia cells) and primary monocyte chemotaxis (70 and 50 %, respectively). BNP did not interfere with MCP1 receptor expression or with calcium. BNP inhibited activation of the cytoskeletal protein RhoA in MCP1-stimulated THP1 (70 %). Finally, BNP failed to inhibit MCP1-directed motility of monocytes from patients with hypertension (n=10) and HF (n=6) suggesting attenuation of this anti-inflammatory mechanism in chronic heart disease. We provide novel evidence for a direct role of BNP/NPRA in opposing human monocyte migration and support a role for BNP as a cardio-protective hormone up-regulated as part of an adaptive compensatory response to combat excess inflammation.

Keywords BNP · NPRA · RhoA · Monocyte chemotaxis · Calcium · Hypertension · Heart failure

INTRODUCTION

Heart failure (HF), regardless of the underlying cause—systolic or diastolic dysfunction, is driven by the maladaptive response to injurious insults, such as pressure overload or myocardial infarction. Inflammation is a crucial component of the global response to injury and develops in the heart early after an acute myocardial injury or stress. Inflammation is known to play a major role in the process of HF and is caused by the up-regulation of inflammatory mediators in the blood and the failing myocardium [1–3]. During the progression of hypertensive heart disease and HF, there is an infiltration of inflammatory cells, particularly monocytes and macrophages, in the perivascular regions of the heart. These cells are recruited by gradients of chemoattractants, such as monocyte chemoattractant protein (MCP)1, which extend from the tissue to the peripheral circulation and cause migration into the affected cardiac tissue. Distinctive inflammatory cell accumulation has been detected in perivascular regions of pressure-overloaded rat hearts [4, 5] as well as in endomyocardial biopsies from subjects with HF [6]. The increased accumulation of monocytes and macrophages subsequently leads to detrimental cardiac effects, such as myocardial fibrosis, hypertrophy, impaired cardiac function and HF [4–11]. Following from these findings, novel anti-cytokine and immunomodulatory therapies for HF have been developed and tested [12–15].

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Whilst some have shown benefit, further development of these approaches is required, perhaps tailored to the cause, course and type of HF.

Therefore a more in-depth understanding of the inflammatory processes and the accompanying early events in cardiac injury will aid the optimisation of immunomodulatory management of HF.

An early marker that is widely regarded as a reliable indicator of cardiac stress is the cardiac-derived hormone B-type natriuretic peptide (BNP). BNP's known functions include relief of cardiac stress via augmentation of diuresis, natriuresis and vasodilation; inhibition of renin; regulation of vasopressin and aldosterone secretion; suppression of cardiac sympathetic nervous system activity; and inhibition of cardiac cell growth and proliferation [16–18]. High BNP levels have been shown to strongly correlate with hypertension, myocardial infarction, chronic HF [19, 20] and with the severity of systolic and diastolic dysfunction [21, 22]. In addition, modest elevations in peripheral BNP in asymptomatic hypertensive (HTN) patients correlated with significant changes in central (coronary sinus (CS)) BNP, CS inflammatory markers, and CS markers of collagen types I and III turnover and were associated with sub-clinical evidence of cardiac remodelling, inflammation and extracellular matrix alterations [23]. Thus, BNP is currently used as a diagnostic and prognostic biomarker for HF in the clinic [24]. In therapy, human recombinant BNP, also known as Nesiritide, is used on top of standard care including diuretic therapy and has been shown to provide clinical benefit for acutely decompensated HF patients [25], although its efficacy in acute HF has recently been questioned [26]. However, other BNP-based therapeutics in HF including chronic subcutaneous BNP protein therapy [27] and vasopeptidase inhibition [28] still hold promise.

Transgenic animal studies have implied a role for the BNP/BNP receptor (natriuretic peptide receptor A (NPRA)) system in regulating proliferation and fibrotic processes in the heart [29–31]. However, only recently a link between early cardiac inflammatory events and BNP has been suggested. Animals with impaired BNP/NPRA pathway had enhanced cardiac pro-inflammatory cytokine gene expression (tumor necrosis factor α (TNF-α), interleukin (IL)-6 and MCP1) linked to elevated nuclear factor kappa B activity as well as increased transforming growth factor β expression, cardiac fibrosis and HF [31, 32]. Furthermore, elevated levels of BNP correlated with markers of inflammation and fibrosis (IL-6, TNF-α, C-reactive protein and left ventricular (LV) end-diastolic dimensions) in patients with idiopathic LV dysfunction [33], chronic HF [34] and in HTN patients at risk of developing diastolic HF [35]. BNP levels rise not only as a result of increased cardiac tension but also due to impaired BNP/NPRA signalling and natriuretic peptide hypo-responsiveness, which develops in chronic HF [36]. In fact, a recent study by Macheret et al. also demonstrated the existence of an impaired production and/or release of the precursor for BNP, the N-terminal fragment, as well as the mature BNP peptide in the very early stages of hypertension, with a significant elevation only in stage 2 hypertension [37] again supporting the concept of impaired BNP/NPRA responses in chronic hypertension and HF.

Taken together, these data show a strong association between BNP and active inflammation in the diseased heart and implicate the BNP/NPRA pathway in the regulation of inflammatory processes. Whilst BNP and NPRA knockout mice imply an anti-inflammatory role for this peptide, BNP overexpression in mice has been shown to facilitate neutrophil infiltration and cardiac matrix metalloproteinase 9 expression after acute myocardial infarction, suggesting a role in acute inflammation, extracellular matrix remodelling and wound healing [38]. The relevance of this in chronic disease is unclear as a significant role for neutrophils in models of pressure overload has been questioned; monocytes and not neutrophils were seen in the hypertrophied heart where they contributed to wound repair and fibrosis [8]. To date, no studies have evaluated the effects of BNP in human monocytes. Considering the importance of inflammation and myocardial monocyte infiltration for the pathogenesis of chronic heart disease, it is also important to assess the role of BNP on monocyte motility.

We thus hypothesised that the BNP/NPRA pathway, in addition to its anti-hypertrophic [39, 40], fibro-protective [29, 41] and cytoprotective anti-ischaemic [42] cardiac functions, may also be able to modulate the inflammatory response via direct effects on monocytes and monocyte function. Our study aimed to evaluate the expression of the BNP receptor in human monocytes and to study the effects of BNP on MCP1-mediated monocyte motility and migration, and cellular processes which may contribute including MCP1–MCP1 receptor (CCR2) turnover, activation of the cytoskeletal protein RhoA and calcium regulation.

MATERIALS AND METHODS

Cell Lines and Primary Cells

THP1 cells (human acute monocytic leukemia cell line) were cultured in RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 10 % fetal calf serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin and maintained at a density of $0.2–1.0\times10^6$ cells/ml. Primary human monocytes were isolated from venous blood of healthy volunteers or patients in two steps with Dynabeads MyPure Monocyte Kit 2 for untouched human cells (Invitrogen). The purity of

monocyte populations was confirmed via assessment of CD14 expression by flow cytometry and was approximately 85 %. All procedures for work with blood samples from healthy volunteers were approved by the local research ethics committee (UCD, human research ethics committee).

Patients

All patients gave written informed consent to participate in the study. The study protocol was approved by the ethics committee at St. Vincent's University Hospital and conformed to the principles of the Helsinki Declaration. The study population consisted of 16 Caucasian HTN patients: ten asymptomatic HTN patients and six patients with clinical symptoms of HF. All subjects had normal LV systolic function and preserved LV ejection fraction (LVEF, >45 %), i.e. diastolic HF. Exclusion criteria included history of (auto)immune disorders; cardiac diseases (include valve stenosis, ventricular arrhythmias or cardiomyopathy of any kind); renal insufficiency; hepatic dysfunction; conditions, known to alter collagen turnover including chronic liver disease, connective tissue disease, metabolic bone diseases and malignancies; evidence of infection; severe anaemia; and recent surgery or physical trauma (<6 months). Two-dimensional echocardiography imaging, targeted M-mode, and Tissue Doppler Imaging were performed using standard techniques to assess LV function. Peripheral BNP levels were obtained as part of the routine clinical examination. Demographic data and characteristics of the two study groups are shown in Table 1. There were no significant differences between the groups apart from age (p=0.002) and peripheral BNP (p=0.0003), which were significantly associated with disease status. Both parameters have been well associated with LVDD and HF, and BNP is a well-established clinical diagnostic and prognostic marker for diastolic dysfunction and HF [43–45]. High creatinine consistent with impaired renal function and kidney hypoperfusion was detected in HF patients. Sixty per cent of the patients in the HTN group had evidence of LVDD, but all patients were asymptomatic. All HF patients had LVDD and symptoms of HF (New York Heart Association functional classes II–III).

Cyclic GMP Enzyme Immunoassay

Correlate-EIA cyclic GMP enzyme immunoassay kit (Assay Designs) was used for quantitative determination of intracellular cGMP levels in BNP- vs. non-BNP-treated THP1. The acetylated version of the assay was used. The samples were prepared and assayed according to the manufacturer's instructions. THP1 cells were treated with hBNP-32 (American Peptide Company, Sigma) at concentrations of 1 (0, 1, 3, 5 or 10 min) or 0.1 μM (0 or 5 min) and lysed in 100 μl of the provided lysis buffer. Following the immunoassay, the optical density for each sample was read at 405 nm with correction at 570 nm with Spectramax190 (Molecular Devices).

RNA Isolation and cDNA Synthesis

RNA was prepared using NucleoSpin® RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with addition of random hexamer primer and SuperScript II RNase reverse transcriptase (Invitrogen) according to instructions from the manufacturer. cDNA was synthesised by

Table 1 Demographic, medical and biochemical characteristics of hypertensive patients with and without clinical evidence of heart failure

Variables	HTN (n=10)	HF (n=6)	p values
Age (years)	65±6	79±8	0.002
Male (%)	6 (60 %)	4 (67 %)	1.00
Body mass index (kg/m^2)	30±5	30±5	0.81
BNP (pg/ml)	15 (8/39)	231 (159/436)	0.0003
White cell count (10^9/l)	6.7±3	6.8±2	0.98
Haemoglobin (g/dl)	13.3±1.3	12.2±1.5	0.15
Platelets (10^9/l)	236±47	217±66	0.53
Creatinine (μmol/l)	75±16	97±20	0.029
Cholesterol (mmol/l)	4.4±1.2	4.6±1.0	0.82
LVDD (%)	6 (60 %)	6 (100 %)	0.23
Ejection fraction (%)	62±6	64±10	0.68
LV mass (gm)	216±55	265±66	0.19
LVMI (gm/m^2)	104±27	138±30	0.11
LAVI (ml/m^2)	28±8	69±38	0.02
e′ (cm/s)	7.9±1.4	9.7±4.8	0.39
E/e′	8.7±3.6	14.5±9.5	0.20

Values are presented as mean±SD or median (25th/75th percentiles) for normally and non-normally distributed continuous variables respectively and frequencies and percentages (N, in percent).

LVMI left ventricular mass index, *LAVI* left atrial volume index, *E/e′* ratio of mitral early diastolic flow velocity over tissue Doppler mitral annular lengthening velocity

incubations at 25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min (Eppendorf, Mastercycler gradient).

Western Blot

Equal amounts of cell lysate from human THP1, primary PBMC, lymphocytes, monocytes and human cardiac tissue lysate (quantified with a BCA protein assay kit (Pierce)) were run on 7–12 % polyacrylamide gels and transferred to a PVDF membrane. Non-specific binding was blocked by incubation in TBS-T with 5 % milk. Membranes were incubated with rabbit anti-NPRA (1:1,000, Abcam) or rabbit anti-RhoA (1:1,000, Santa Cruz)) followed by goat anti-rabbit HRP (1:20,000, Abcam). Luminescent signals were detected following exposure of the membrane to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Cardiac tissue lysate served as a positive control for NPRA protein.

PCR for NPRA

PCR for NPRA in THP1, primary PBMC, lymphocytes, monocytes and ventricular human cardiac fibroblasts (VHCF) was performed using Hot-Goldstar Red Taq polymerase (Eurogentec) along with human NPRA-specific forward and reverse primers (450 and 300 nM, respectively; MWG Biotech) and 1 μl cDNA template in a 20-μl reaction. The sequences of the used primers were: cgcaaaggccgagttatcta (NPRA F2), gtaacagttcccccatgtgc (NPRA R1) and aacgtagtcctccccacaca (NPRA R2). A 98-bp amplicon was generated using NPRA F2/R2 primers by incubations at 95 °C for 10 min, 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s (for 35 cycles) and 72 °C for 10 min (Eppendorf, Mastercycler gradient). VHCF served as a positive control. The products were run on a 2 % agarose gel. NPRA F2/R1 primers were used (annealing temperature, 60 °C) to generate a 415-bp amplicon used for NPRA sequencing.

Migration Assays

Non- or BNP-treated (1 μM, 1 h, 37 °C) THP1 or primary monocytes (1×10^5 cells) were applied to the apical compartment of 24-well polycarbonate membrane transwell inserts (Corning Incorporated). THP1 monocytes were serum-starved overnight prior to migration and assays were performed in serum-free medium. Primary monocytes were isolated from fresh blood and directly used in migration assays, also in serum-free conditions. A pore diameter of 5 μm was used for THP1 and 3 μm for primary monocyte migration. MCP1 (R&D Systems) was applied in concentrations of 5, 15 or 25 ng/ml (25 ng/ml for patient-derived primary monocytes) to the basal compartment of the system. The cells were incubated for 3 h (THP1) or 1.5 h (all primary monocytes) at 37 °C. The cells which migrated to the basal side of the transwell insert were fixed, permeabilised and stained with DAPI (c=0.5 μg/ml, Sigma). The mean cell count from ten random fields (at ×10 magnification) was used for the analysis.

CCR2 Surface Staining

THP1 cells were cultured in serum-free medium overnight and treated with 1 μM BNP for 1 h prior to the experiment. The cells (2×10^5 cells/condition) were stimulated with MCP1 (25 ng/ml) for 0, 15, 30 or 60 min and washed in ice-cold PBS/0.5 % BSA. Non-specific binding was blocked by incubation with FcR blocking reagent (Miltenyi Biotec) for 15 min at 4 °C and was followed by staining with CCR2-PE or IgG2b-PE antibodies (RnD Systems) for 45 min at 4 °C. Surface expression of CCR2 was determined by flow cytometry (Accuri C6); 100,000 live cells were assessed in each sample.

RhoA Pulldown Assay

RhoA pulldown assays were performed with the Rho activation assay Biochem Kit (Cytoskeleton) according to the manufacturer's instructions. THP1 cells (2.5×10^6 cells/ml) were cultured in serum-free medium overnight, treated with/without BNP (1 μM, 1 h, 37 °C) and stimulated with MCP1 (25 ng/ml; 0, 15 and 30 min; 37 °C). Cell lysates were prepared and equivalent amounts of protein (300 μg) were added to 50 μg of rhotekin-RBD beads. Following incubation, the beads were pelleted, washed, boiled at 95 °C for 2 min in ×2 Laemmli sample buffer with β-mercaptoethanol and analysed by western blot. Along with the bead samples, whole protein lysates (10 μg) from each sample were run and used as controls for the total amount of RhoA (tRhoA).

Calcium Measurements with Fluo-4 AM

THP1 cells were cultured in serum-free medium overnight, treated with BNP (1 μM, 1 h, 37 °C), and loaded with 1 μM Fluo-4 AM (Biosciences) for 30 min at 37 °C. Following two washes, the cells were resuspended in serum-free medium which has been treated or not with 1 mM ethyleneglycolbis(2-aminoethylether)tetraacetic acid (EGTA; Lennox). Changes in intracellular calcium levels were determined by flow cytometry by measuring the fluorescence intensity of fluo-4 AM (Accuri C6). Total acquisition time was 5 min. The cells were stimulated with MCP1 (25 ng/ml) or BNP (1 μM) during the acquisition approximately 1 min from the beginning of the measurement.

Statistical Methods

Data across two groups were statistically analysed using unpaired *t* test or Mann–Whitney U test for normally and non-normally distributed data, respectively. One-way ANOVA/Repeated measures test with Bonferroni multiple comparisons test or Kruskal–Wallis test with Dunns multiple comparisons test were used, where appropriate, for comparisons across more than two groups. All tests were two-sided with $p<0.05$ as the criterion for significance. Data are reported as means±SEM. GraphPad Prism 4.03 software was used for analysis.

RESULTS

Human Monocytes Express a Functional Receptor for BNP–NPRA

Expression of the NPRA gene was detected in THP1 monocytes and in peripheral blood mononuclear cells (PBMC) (Fig. 1a). Culture of PBMC for 3 h on polystyrene, in the presence of serum allowed separation of PBMC into two fractions: adhesion-enriched monocyte fraction and a non-adherent lymphocyte fraction [46]. Expression of NPRA was identified exclusively in the monocyte fraction. VHCF have previously been shown to express natriuretic peptide receptors including NPRA [47] and were therefore used as positive controls. To confirm the specificity of the PCR, a larger fragment of NPRA was amplified and sequenced. The product showed 100 % sequence identity with the human NPRA gene (data not shown). NPRA expression was confirmed also at the protein level (Fig. 1b) in whole cell lysates of THP1 cells, PBMC, adhesion-enriched monocytes and whole cardiac tissue but not in non-adherent PBMC, i.e. lymphocytes.

Incubation of THP1 cells with BNP slightly increased intracellular cGMP levels (1.6-fold) following culture with 0.1 μM BNP (Fig. 1c, left). A robust (3.7-fold) cGMP accumulation was detected when 1 μM BNP was used. A time-course of BNP treatment (1 μM, 0 to 10 min) showed maximal induction of cGMP when THP1 cells were stimulated with BNP for 5 min (3.3-fold, $p<0.05$) (Fig. 1c, right). The cGMP response was transient and levels rapidly decreased to basal following 10 min of BNP stimulation (0.9-fold).

BNP Inhibits MCP1-Induced Monocyte Chemotaxis

Non- and BNP-treated THP1 and primary monocytes were used to study migratory responses to MCP1 (Fig. 2a, b, respectively). Monocyte migration to MCP1 at three different concentrations (5, 15 and 25 ng/ml) was performed. At a physiologically relevant MCP1 concentration (25 ng/ml), pre-treatment of monocytes with BNP caused significant inhibition of MCP1-induced chemotaxis. THP1 migration was reduced by 70 % ($p<0.01$), and a 50 % inhibition was seen in primary monocytes ($p<0.05$).

The Inhibitory Effect of BNP Is Independent of CCR2

To examine whether BNP can influence MCP1 receptor expression, we compared CCR2 levels in BNP- vs. non-treated THP1, stimulated with MCP1 (0, 15, 30 and 60 min) using flow cytometry (Fig. 3). Compared with a non-treated control, BNP treatment alone did not have an effect on CCR2 surface expression. Stimulation with MCP1 resulted in reduced surface expression of the receptor, possibly via CCR2 internalisation. Compared with control, mean CCR2 levels were reduced to 67 % after 15 min ($p<0.05$), 58 % after 30 min ($p<0.01$) and 55 % after 60 min ($p<0.01$) of MCP1 treatment. Pre-treatment with BNP did not have an effect on CCR2 levels in control or MCP1-treated THP1.

BNP treatment reduces RhoA activation in MCP1-stimulated THP1 monocytes

Pulldown assays were performed to investigate the effects of BNP on the activation status of the cytoskeletal regulatory protein RhoA. MCP1 is a known inducer of monocyte adhesion and chemotaxis via pathways involving activation of RhoA/ROCK [48]. Thus, MCP1-induced activation of RhoA served to define upper thresholds of RhoA activation in the pulldown assays. Non- and BNP-treated THP1 monocytes were stimulated with MCP1 (25 ng/ml) for 30 min and RhoA-GTP in the lysates was pulled down and quantified by western blot (Fig. 4). A representative western blot result is shown in Fig. 4a and densitometry analysis with combined data from three independent experiments is presented in Fig. 4b. A trend for increased RhoA-GTP was observed when the cells were treated with BNP alone but this did not prove statistically significant. Stimulation with MCP1 increased RhoA-GTP levels approximately 5.4-fold ($p<0.05$). Treatment with BNP prior to stimulation with MCP1 almost completely abolished RhoA activation observed at the peak of MCP1 induction (down to 1.7-fold) ($p<0.05$).

BNP treatment induces Ca^{2+} influx in THP1 monocytes, but does not interfere with MCP1-induced accumulation of intracellular calcium

Accumulation of intracellular calcium in THP1 monocytes exposed to BNP and MCP1 was studied to attribute or exclude a role for BNP in the regulation of calcium.

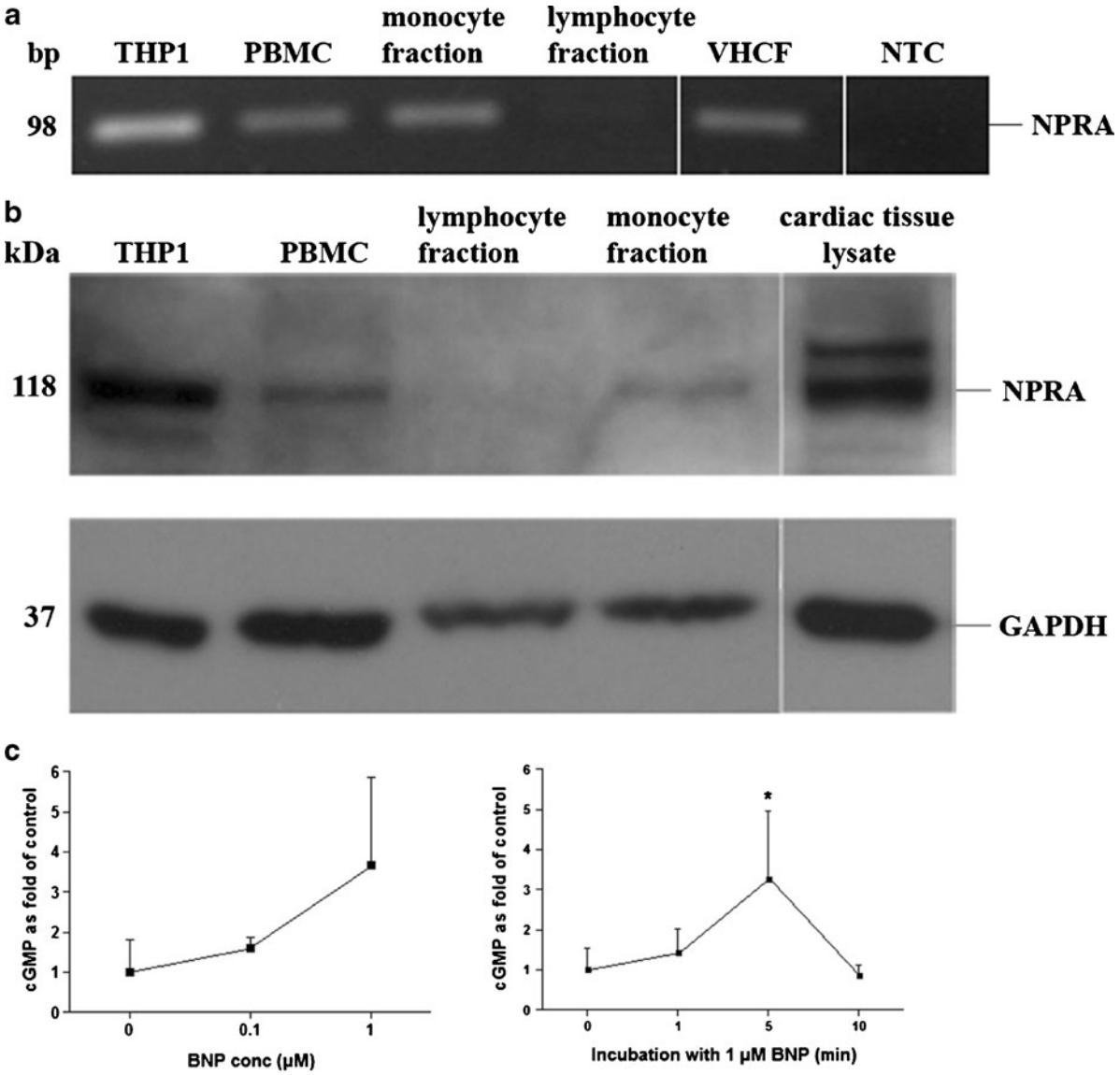

Fig. 1 Identification of a functional NPRA in human monocytes. **a** NPRA mRNA expression in THP1, PBMC, primary adherent monocyte and non-adherent lymphocyte PBMC fractions and ventricular human cardiac fibroblasts (*VHCF*). VHCF were used as a positive control for NPRA expression. *NTC* non-template control. **b** NPRA protein expression in THP1, PBMC, lymphocyte and monocyte PBMC fractions, primary monocytes and cardiac tissue lysate as a positive control (all 40 μg). Primary monocyte and lymphocyte PBMC were obtained by culture of PBMC in complete medium for 3 h at 37 °C and subsequent collection of adherent (monocytes) and non-adherent (lymphocytes) cells. **c** Intracellular cGMP levels in BNP-treated THP1: fold induction of cGMP following incubation of THP1 with 0, 0.1 and 1 μM BNP (5 min; *left graph*, $n=2$); Time-course (0 to 10 min) of cGMP levels in THP1 treated with 1 μM BNP (*right graph*, $n=5$). *$p<0.05$, level of significance. *Bp* base pairs, *kDa* kilodalton

Stimulation of non-treated THP1 with MCP1 increased intracellular calcium levels by 55 % ($p<0.01$) in the presence of extracellular calcium in the medium (Fig. 5a). The magnitude of the MCP1-induced calcium signal was similar in cells, treated with BNP, however, the induction was no longer significant due to an increase in baseline intracellular calcium levels (30 %, $p=0.045$).

Next, the source of intracellular calcium in BNP-treated THP1 monocytes was investigated. THP1 cells were resuspended in RPMI medium or RPMI serum-free medium deprived of calcium via treatment with the chelating agent EGTA (1 mM). Whilst in the presence of extracellular calcium BNP increased intracellular calcium levels, as described above (30 %, $p=0.045$), calcium chelation from the medium completely abolished this effect (Fig. 5b). This suggested that BNP may act on calcium channels in the plasma membrane of THP1 monocytes to increase calcium influx. To follow this hypothesis, the above experiment (Fig. 5a) was repeated with cells cultured in calcium-free RPMI medium prior

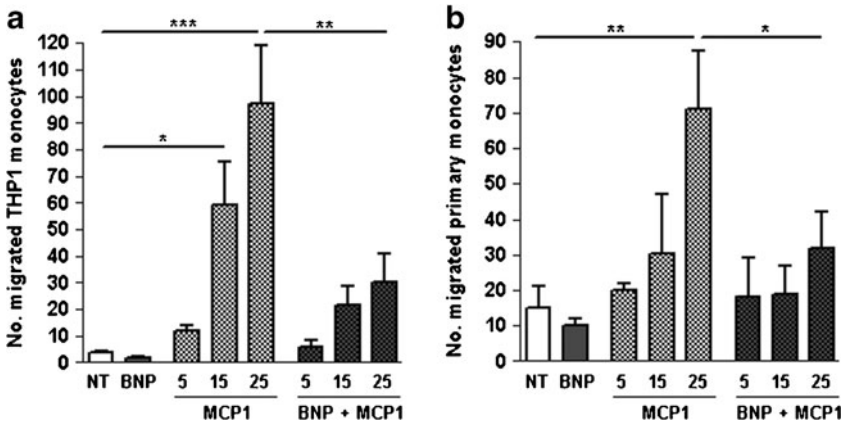

Fig. 2 Transwell migration assays with human monocytes. Migration of THP1 (n=6) **(a)** and primary monocytes (n=4) **(b)** in response to culture with BNP and/or MCP1 (5, 15 and 25 ng/ml). Migration time was 3 h for THP1 and 1.5 h for primary monocytes. *p<0.05, level of significance. *NT* non-treated control

to stimulation with MCP1 (Fig. 5c). In this set-up, BNP no longer elevated baseline calcium levels. Stimulation with MCP1 induced a 2.2-fold increase in calcium in non-treated (p<0.05) and a comparable 2.1-fold increase in calcium was seen in BNP-pre-treated cells (p<0.05).

BNP Does Not Affect MCP1-Directed Migration of Monocytes of Patients with Hypertension and HF

Migration assays were performed to assess the effects of BNP on basal and MCP1-induced monocyte motility in

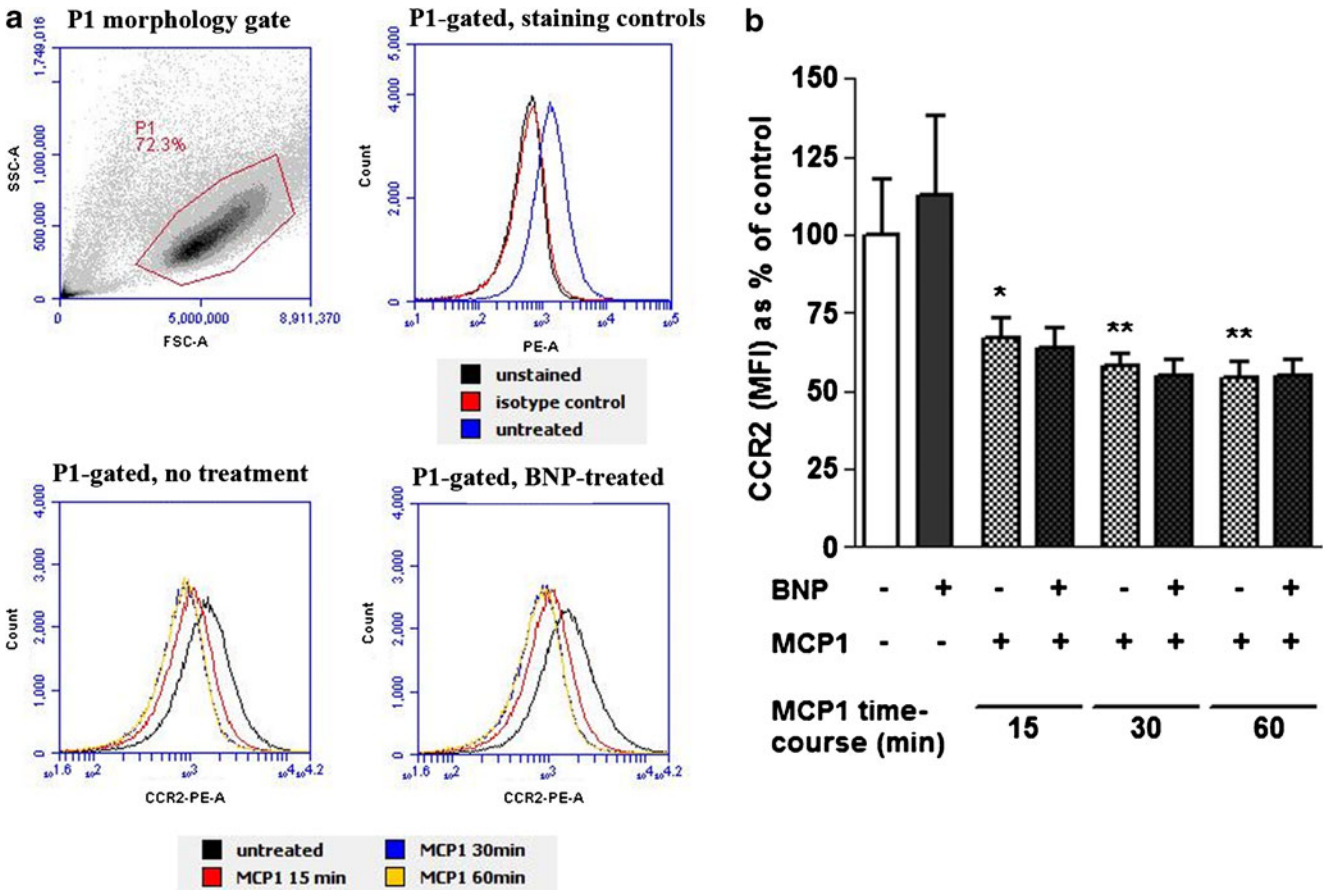

Fig. 3 Effect of BNP on monocyte CCR2 surface expression. THP1 cells were treated with BNP, stimulated with MCP1 (0 to 60 min) and stained with an anti-CCR2-PE antibody for detection of CCR2 surface expression by flow cytometry (n=4). **a** Flow cytometry plots representing: forward/sideward scatter (*FSC/SSC*) profile of cells with monocyte live cell morphology gate (*P1*; *upper left*); histogram overlay of P1-gated cells showing staining controls: unstained, isotype control and CCR2-PE-stained, untreated THP1 cells (*upper right*); histogram overlays (in P1 gate) showing CCR2-PE-stained non- (*lower left*) and BNP-treated (*lower right*) THP1 cells stimulated with MCP1 for 0, 15, 30 and 60 min. **b** Collective data from four independent experiments. *MFI* mean fluorescence intensity. *p<0.05, significant p values compared with the untreated control (*white bar*)

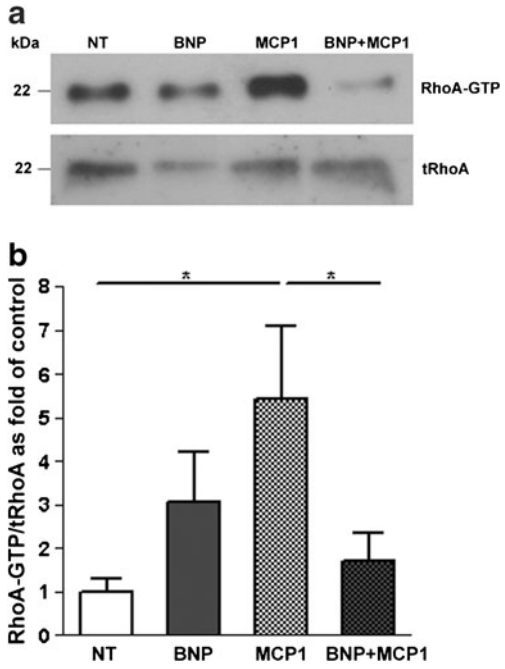

Fig. 4 RhoA pulldown assays in THP1 monocytes. **a** Representative blots of RhoA-GTP and tRhoA in BNP-treated THP1 monocytes stimulated with MCP1 as identified by Western blot. **b** Respective densitometry data showing fold RhoA-GTP normalised to tRhoA in each sample (n=3). *p<0.05, level of significance. *NT* non-treated control

monocytes obtained from patients with asymptomatic HTN and symptomatic HF (Table 1; Fig. 6). In contrast to its effects in healthy control monocytes, BNP did not block monocyte chemotaxis to MCP1 in patient-derived monocytes and there was even a slight but non-significant tendency for increased migration to MCP1 in BNP-stimulated monocytes both in the HTN and the HF groups. Interestingly, there was also a trend for increased spontaneous hypermotility of monocytes from HF (p=0.054) but not from HTN patients in response to BNP treatment.

DISCUSSION

During HF, BNP levels increase significantly and correlate with disease progression [49]; this is paralleled by increased concentrations of inflammatory cytokines [50]. Surprisingly, little is known about the link between inflammation and BNP, although it is considered that BNP may be up-regulated as part of an adaptive response to compensate for excessive inflammation in HF. Our data reveal novel expression of a functional receptor for BNP–NPRA in human primary monocytes. Although, in a recent study, Pivovarova et al. showed expression of NPRB and NPRC, but not NPRA receptor in peripheral blood monocytes [51], we are convinced that this discrepancy is due to the low levels of this gene in monocytes which makes it difficult to detect. We also showed that BNP can inhibit monocyte motility towards the chemokine MCP1, modulate basal calcium levels within THP1 monocytes and inhibit MCP1-mediated Rho activity. Lastly, in contrast to healthy donor monocytes we show that monocytes derived from patients with asymptomatic hypertension or HF with preserved ejection fraction appear to have lost the inhibitory response to BNP. We thus propose that, in monocytes, the BNP/NPRA pathway has an anti-inflammatory role. However, the pathway may be susceptible to attenuation in patients with chronic disease.

Monocyte migration from the peripheral circulation into an affected tissue, for example during cardiac stress, is a key step in the initiation of inflammatory responses. We showed that BNP treatment strongly attenuates the stimulatory effect of MCP1 on monocytes and almost completely inhibited chemotaxis. BNP has been shown to modulate the production of various pro- and anti-inflammatory mediators and affect the motility of phorbol ester-differentiated THP1 macrophages but no further mechanistic insight has been provided [52]. BNP treatment of PBMC from cardiac transplant recipients has also been shown to reduce the expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1α) and preserve the expression of regulatory cytokines (IL-4, IL-5 and IL-13) [53].

To understand how BNP regulates the process of monocyte migration, we investigated the ability of the peptide to regulate the MCP1 pathway at the receptor level. The MCP1-CCR2 pathway contributes to the inflammatory component of chronic cardiovascular diseases such as atherosclerosis and hypertension-induced vascular inflammation and remodelling [54–56] and blocking the pathway with an anti-MCP1 monoclonal antibody [7] or knocking out MCP1 or CCR2 expression [56, 57] has been shown to ameliorate disease in experimental animal models by reducing the inflammatory burden. MCP1–CCR2 interaction promotes monocyte adhesion, migration into the tissue and monocyte/macrophage differentiation. After activation CCR2 is internalised and lost from the cell surface providing a method of desensitisation. Whilst we had hypothesised that BNP might promote receptor internalisation, we found that it had no effect on surface expression of CCR2 and did not interfere with CCR2 regulation by MCP1.

There are several additional intracellular pathways and mediators of monocyte migration which may be involved in the actions of BNP in monocytes. Both BNP/NPRA and MCP1/CCR2 act through protein kinase G (PKG) and may thus be able to regulate the activation status of its effector

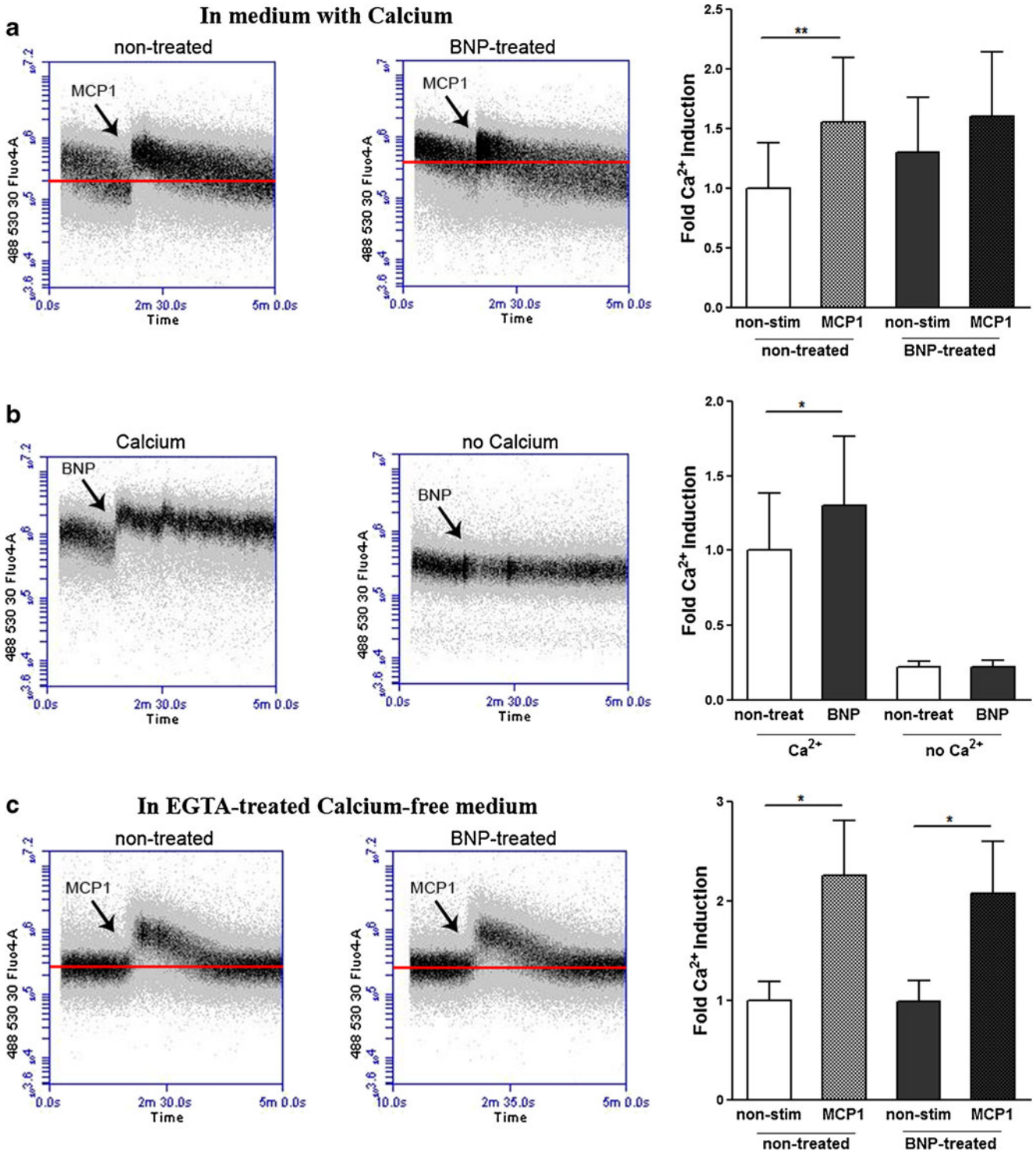

Fig. 5 Intracellular calcium measurements in THP1 monocytes. **a** Induction of calcium in non- vs. BNP-treated THP1, suspended in serum-free culture medium following stimulation with MCP1 (n=4). **b** Calcium induction in THP1, suspended in serum-free culture medium (with/without EGTA treatment) following stimulation with BNP (n=4). **c** Induction of calcium in non- vs. BNP-treated THP1, suspended in EGTA-treated serum-free culture medium following stimulation with MCP1 (n=4). Figures represent intracellular calcium release, reflected by an increase in fluo-4 intensity (*y-axis*) plotted over time (*x*-axis). *Arrows* identify the points of stimulation with BNP or MCP1. *Red lines* indicate average fluo-4 intensity prior to point of stimulation. *p<0.05, significant p values. *Non-treat* non-treated, *non-stim* non-stimulated

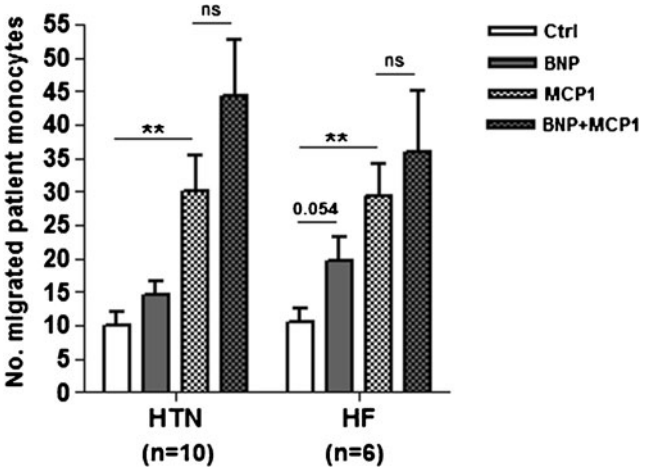

Fig. 6 Patient monocyte migration assays. Migration of HTN (n=10) and HF (n=6) patient-derived monocytes following 1.5 h of culture with BNP (1 μM) and/or MCP1 (25 ng/ml). *Ctrl* non-treated control. **p<0.01, significant p values of treated vs. ctrl in the corresponding patient group. *ns* not significant

protein RhoA. PKG expression has been shown in human monocytes and monocyte-derived macrophages and a role in cGMP-mediated protein processing and cell motility has been anticipated [58]. PKG has been shown to inhibit RhoA signalling and the subsequent activation of downstream effectors of RhoA (i.e. RhoA kinases or ROCKs) by relocating active RhoA from the cell membrane to the cytosol [59]. PKG has also been shown to regulate and inhibit calcium influx in different cells, and also contributes to NO-mediated vasorelaxation [60, 61]. RhoA is a known regulator of actin stress fibers and focal adhesions and can affect cell morphology and motility [62]. We showed that treatment with BNP inhibits MCP1-induced RhoA activation in human monocytes. We also observed a trend for increased basal RhoA activity in BNP-treated cells in the absence of MCP1 stimulation. This likely suggests that BNP regulates RhoA either by modifying the molecular switch of RhoA (RhoA-GDP↔RhoA-GTP) or by switching off RhoA activation, for example by enhancing the intrinsic GTPase activity of the protein leading to the hydrolysis of GTP to GDP. RhoA activation in monocytes promotes migration across endothelial cell layers [63], with RhoA specifically required for monocyte tail retraction during the migratory process [64]. The profound effect of BNP on RhoA activation in MCP1-stimulated monocytes may therefore be a major mechanism by which BNP exerts its inhibitory effects on monocyte migration.

As calcium influx has been implicated in the activation of RhoA in vascular smooth muscle cells [65] and in the regulation of macrophage chemotaxis [66], we also studied accumulation of intracellular calcium in response to MCP1 in BNP- and non-treated THP1 monocytes. Stimulation with MCP1 caused a significant elevation of calcium levels in non-treated cells. Interestingly, in BNP-treated THP1 cells, basal intracellular calcium levels increased rendering the MCP1 response insignificant. The peaks of MCP1-induced calcium accumulation were comparable between BNP- and non-treated cells. In order to assess the mechanism by which BNP elevated basal calcium levels, we depleted calcium in the culture medium and demonstrated that BNP treatment induces an influx of extracellular calcium. BNP appeared to have no effect on the mobilisation of calcium from intracellular stores. These data indicate that whilst BNP may not interfere with the regulation of calcium by MCP1, it may itself regulate calcium influx in monocytes via calcium channels in the plasma membrane of cells.

Although monocyte calcium channels are not very well characterised, transient receptor potential channels (TRPC), a type of store-operated calcium channels, have been reported in several cell types including monocytes and increased monocyte TRPC3 and 5 expression has been associated with cardiac hypertension and hypertrophy [67–70]. Nitrogen oxide, atrial natriuretic peptide and BNP have previously been shown to inhibit TRPC1/3, and TRPC6-operated calcium influx in smooth muscle cells by phosphorylation of the channels, induce vasorelaxation and antagonise hypertrophy [61, 71]. Whilst the findings in smooth muscle cells do not support our findings it is possible that BNP has different effects on calcium channels in monocytes, a topic which has not yet been investigated. In addition, a more complex calcium regulation in monocytes involving multiple calcium channels (e.g. calcium-sensing receptors [72, 73]) should not be ruled out.

Finally, to explore the significance of the observed inhibitory effect of BNP on monocyte motility in a pathologically relevant context, we investigated the migratory properties of monocytes from patients with asymptomatic hypertension with and without clinical symptoms of HF. In contrast to its potent effect in healthy monocytes, in both patient groups there was no effect of BNP on MCP1-driven monocyte motility. In addition, monocytes from HF patients, which have been chronically exposed to high endogenous levels of BNP (>100 pg/ml), showed a trend for increased spontaneous motility in response to BNP treatment (p=0.054). This was not caused by changes in expression of the receptor for BNP–NPRA as no difference in NPRA gene expression was seen between HTN and HF patient monocytes (Fig. S1 in the Electronic Supplementary Material (ESM)). However, it may be related to NPRA internalisation, desensitisation, or degradation as it is known that chronic exposure to an activating ligand (i.e. BNP) reduces hormone-dependant guanylyl cyclase activity of NPRA (reviewed in [17]). This may ultimately result in high circulating BNP levels (seen in HF patients), which may stimulate other monocyte activation pathways. Hypertensive patient monocytes showed a high migratory response to MCP1 which could no longer be

attenuated by BNP. As MCP1 is majorly involved in the recruitment of monocytes to sites of injury in cardiovascular disease (e.g. atherosclerosis [55] and diastolic HF [35]), our finding provides an insight into how excessive inflammation in HTN and HF may occur. The mechanism for this attenuated response is unclear, but it could be related to altered sensitivity to MCP1 or altered PKG activity but not to changes in MCP1 receptor surface levels between HTN and HF patients (see Fig. S2 in the ESM). In fact, a recent study has shown decreased PKG activity in LV biopsy tissue of patients with HF with preserved ejection fraction [74]; whether this relates to peripheral monocytes remains to be seen. In addition, lack of responsiveness of BNP-treated patient monocytes to MCP1 could be due to desensitisation to treatment with exogenous BNP.

Several limitations to this study exist. First, most of our data appear to be based on studies in a monocyte cell line rather than primary cells. However, whilst, for convenience and robustness, we used monocytic cells for basic molecular studies of the BNP pathway in monocytes, all functional studies were performed with primary monocytes as well. Another limitation to the study may be considered the high concentration of BNP which was used (1 μM or 3.47 ng/ml). Indeed, for diagnosis of HF, a cut-off for plasma BNP of 0.03 μM (0.1 ng/ml) in patients with new onset or worsening symptoms is used [75]. However, in patients with chronic HF, peripheral plasma BNP levels of approximately 1 nM are frequently measured [76]. In addition, as BNP is synthesised within the failing myocardium, it is likely that the local tissue BNP levels that are affecting monocyte function in the injured heart far exceed those measured in the peripheral serum. Another limitation of the study is the difference in age between the healthy population (<40 years) and the HTN and HF patients (>60 years) and this needs to be accounted for if direct comparisons between the studied healthy and non-healthy populations are to be performed. Whilst it has previously been demonstrated that aortic expression of CCR2 increases with age in rats [77], it is unknown whether human monocyte responses to MCP1 or BNP differ with age.

In summary, we provide novel evidence for a direct role of the BNP/NPRA pathway in human primary monocytes. BNP was able to inhibit directional monocyte motility, i.e. chemotaxis towards MCP1. This ability was not associated with the regulation of CCR2 expression and turnover, or with MCP1-induced calcium flux and mobilisation but could be related to BNP's ability to antagonise MCP1 activation of RhoA. Our data implicate BNP as an anti-inflammatory agent opposing the recruitment of monocytes to areas of injury and further support the concept of BNP being a cardioprotective hormone which is up-regulated as part of an adaptive compensatory response.

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Conflicts of Interest The authors confirm that there are no conflicts of interest.

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