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RESEARCH PAPER

Nitric oxide generation by the organic nitrate NDBP attenuates oxidative stress and angiotensin II-mediated hypertension

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BACKGROUND AND PURPOSE

NO deficiency and oxidative stress are crucially involved in the development or progression of cardiovascular disease, including hypertension and stroke. We have previously demonstrated that acute treatment with the newly discovered organic nitrate, 2-nitrate-1,3-dibuthoxypropan (NDBP), is associated with NO-like effects in the vasculature. This study aimed to further characterize the mechanism(s) and to elucidate the therapeutic potential in a model of hypertension and oxidative stress.

EXPERIMENTAL APPROACH

A combination of *ex vivo*, *in vitro* and *in vivo* approaches was used to assess the effects of NDBP on vascular reactivity, NO release, NADPH oxidase activity and in a model of hypertension.

KEY RESULTS

Ex vivo vascular studies demonstrated NDBP-mediated vasorelaxation in mesenteric resistance arteries, which was devoid of tolerance. *In vitro* studies using liver and kidney homogenates revealed dose-dependent and sustained NO generation by NDBP, which was attenuated by the xanthine oxidase inhibitor febuxostat. In addition, NDBP reduced NADPH oxidase activity in the liver and prevented angiotensin II-induced activation of NADPH oxidase in the kidney. *In vivo* studies showed that NDBP halted the progression of hypertension in mice with chronic angiotensin II infusion. This was associated with attenuated cardiac hypertrophy, and reduced NADPH oxidase-derived oxidative stress and fibrosis in the kidney and heart.

CONCLUSION AND IMPLICATIONS

The novel organic nitrate NDBP halts the progression of angiotensin II-mediated hypertension. Mechanistically, our findings suggest that NDBP treatment is associated with sustained NO release and attenuated activity of NADPH oxidase, which to some extent requires functional xanthine oxidase.

Abbreviations

ANG II, angiotensin II; DHE, dihydroethidium; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; NDBP, 2-nitrate-1,3-dibuthoxypropan; NTG, nitroglycerin; PE, phenylephrine; XO, xanthine oxidase

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Tables of Links

TARGETS
Aldehyde dehydrogenase
Xanthine oxidase (XO)

LIGANDS		
ACh	cGMP	Nitric oxide (NO)
Allopurinoll	Febuxostat	Nitroglycerin (NTG)
Angiotensin II	IBMX	Phenylephrine
Ascorbic acid	Nicorandil	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Cardiovascular disease is a multifactorial disorder associated with both metabolic and renal disorders. Hypertension *per se* is a major risk factor, which increases both morbidity and mortality. Further experimental and clinical studies are needed to better understand the pathological mechanisms and to develop novel therapeutic drugs. There is emerging evidence from a number of studies suggesting that oxidative stress and NO deficiency have an important role in the development or progression of cardiovascular disease and its complications. Therefore, new strategies to increase NO signalling, or reduce oxidative stress, are suggested to have therapeutic potential in treating cardiovascular disease, including hypertension (Lundberg *et al.*, 2015). Among the problems with classical NO donors and organic nitrates are unfavourable pharmacokinetics (e.g. short $t_{1/2}$, unspecific location for NO release) and development of tolerance with repeated dosing (Munzel *et al.*, 2005; Omar *et al.*, 2012; Lundberg *et al.*, 2015). Recently, a glycerine-derived organic nitrate, the 2-nitrate-1,3-dibuthoxypropan (NDBP), was synthesized. Our previous studies in rats showed that this newly discovered compound induced vasorelaxation in pre-constricted resistance vessels and acutely reduced blood pressure (BP) in both normotensive and hypertensive rats (Franca-Silva *et al.*, 2012a,b). Pharmacological studies suggested that NDBP causes vasodilatation through NO generation and activation of the sGC/cGMP/PKG pathway. However, previous studies were focused on the acute effects of NDBP, and there is a lack of tolerance studies and chronic assessments on cardiovascular function. Therefore, the present study was aimed to further characterize the underlying mechanism(s) of NDBP and to investigate its therapeutic potential in an *in vivo* model of renal hypertension that is closely associated with activation of NADPH oxidase (NOX) and oxidative stress (Griendling *et al.*, 1994; Kawada *et al.*, 2002; Crowley *et al.*, 2005; Crowley *et al.*, 2006). It was hypothesized that sustained NDBP-mediated NO generation may attenuate NOX-derived superoxide production and slow down the progression of hypertension in mice with chronic angiotensin II (ANG II) infusion.

Methods

Animals and treatments

The scheme for the *in vitro* and *in vivo* study design is shown in Supporting Information Figure S1.

- Part I: For *in vitro* characterization of NDBP regarding vascular relaxation, NO release, and modulation of NOX activity, tissues from adult Wistar rats ($n = 11$) were used.
- Part II: To investigate the therapeutic potential of NDBP in a model of renal hypertension, *in vivo* experiments were performed using C57BL/6 J mice ($n = 24$) treated with a slow pressor infusion of ANG II (400 $ng \cdot kg^{-1} \cdot min^{-1}$) and simultaneously with NDBP (40 $mg \cdot kg^{-1} \cdot day^{-1}$, i.p.). Animals were housed under temperature- and humidity-controlled conditions with a 12 h light/dark cycle and free access to standard chow and water. After the functional studies had been completed, the animals were anaesthetized, and tissues and blood were collected for later analysis.

Preparation of NDBP and NTG. NDBP was dissolved in a mixture of saline solution and cremophor and diluted to the desired concentrations with saline as previously described (Franca-Silva *et al.*, 2012a). In functional studies, the final concentration of cremophor never exceeded 0.01% and had no effect when tested in control preparations *in vitro* or *in vivo* (Supporting Information Figures S2 & S5). Nitroglycerin (NTG) (Abcur AB, 1 $mg \cdot mL^{-1}$, Helsingborg, Sweden) was dissolved in saline and diluted to the desired concentrations with saline.

Part I: ex vivo and in vitro characterization of NDBP

Rats were anaesthetized by subjecting them to spontaneous inhalation of 2–2.5% isoflurane (Forene®, Abbot Scandinavia AB, Solna, Sweden), and renal cortex, liver and mesenteric arteries were collected for analyses. The animals were then killed by injection of KCl.

Tolerance test to NDBP in isolated mesenteric artery vessels. To evaluate tolerance to NDBP in isolated vessels, the cranial mesenteric artery of rats was isolated, placed in Tyrode solution (composition in mM: NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 10.0; and glucose, 5.6) and dissected in order to remove any adhering tissue. The endothelium was removed by rubbing the intimal surface of the vessels using a special type of sterile cotton swab. Rings of 1–2 mm were obtained and placed in physiological Tyrode solution, maintained at 37°C, gassed with carbogenic mixture (95% O_2 and 5% CO_2) and kept at

pH 7.4. All preparations were stabilized under a resting tension of 0.75 g for 1 h, and the solution was replaced every 15 min in order to prevent the accumulation of metabolites (first stabilization). The force of contraction was isometrically recorded by a tension transducer (PowerLab™, ADInstruments, MA, USA), as described previously (Liu *et al.*, 2015). Tissue viability was verified by the presence of a contraction to phenylephrine (PE, 10 μM) added to the bath, and successful removal of the endothelium was verified by the absence of a response to ACh (10 μM), as previously described (Franca-Silva *et al.*, 2012a). In short, if the relaxation to ACh was higher than 90%, the vessel was considered to have an intact endothelium. If relaxation was less than 10%, rings were considered as without endothelium. Rings presenting relaxation between 90 and 10% were discarded. In the first protocol, the preparations were exposed to (i) NDBP (100 μM); (ii) cremophor (100 μM); or (iii) kept untreated (control) for 60 min. After the exposure period, the rings were subjected to a another stabilization (second stabilization). Mesenteric rings were precontracted using PE (10 μM). After the contraction plateau was reached, NDBP (10^{-8}–$10^{-3.5}$ M) was added cumulatively to the organ bath in order to obtain a concentration-response curve. In separate rings, similar experiments were performed using NTG (10^{-12}–10^{-4} M) with or without previous exposure to NTG (100 μM).

Direct measurement of NO. Samples of renal cortex and liver were homogenized in ice-cold PBS with zirconium oxide beads (0.5 mm) using the bullet blender (Next Advance, Inc. Stockholm, Sweden). The homogenate was centrifuged at 4°C for 20 min at 2000 *g*. For measurement of basal NO production, PBS was incubated for 10 min, in the presence or absence of tissue homogenate. Then, NDBP or NTG was administered (3.6 mM), and its effect on NO production was quantified for 60 min. In additional experiments, NDBP-mediated NO formation was investigated in liver and kidney homogenates after inhibition of xanthine oxidase (XO) with febuxostat (1 μM) (Selleckchem, Rungsted, Denmark). Dose- and time-responses to NDBP were assessed using liver homogenates. The sample solution was maintained constantly under rotation at 37°C, at pH 7.4. NO measurement was performed in real time using nitrogen gas in line coupled to a chemiluminescence NO analyser (ECO Physics, CLD 77 AM, Switzerland). NO produced in the sample solution was conducted through a sensor transmitting a signal to the acquisition and quantification software (AcqKnowlege, 12.0). Production of NO was detected as parts-per-billion, and AUC was calculated for the different time periods.

NOX activity. NADPH-mediated formation of superoxide was measured using a lucigenin-dependent chemiluminescence technique. Rat renal cortex and liver samples were homogenized as described above. The fresh homogenates were diluted in PBS and incubated with different concentrations of NDBP (10 μM, 50 μM, 0.1 mM, 1 mM), NTG (10 μM, 50 μM, 0.1 mM, 0.5 mM, 1 mM, 3.6 mM) or vehicle (PBS) for 15 min at 37°C. Then, lucigenin was added (final conc. 5 μM), and after another 10 min at 37°C, the measurements were started by injection of NADPH (100 μM) into the reaction tubes. Additionally, the effect of NDBP (100 μM) on NOX activity was evaluated in the presence of either an XO inhibitor (febuxostat, 30 nM, pretreatment for 15 min before the addition of NDBP) or ANG II (1 μM, pretreatment for 15 min before the addition of NDBP). Superoxide production was recorded by measuring lucigenin-chemiluminescence every 3 s for 3 min with the AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany); the chemiluminescence signal was normalized to the amount of protein in the same sample (Protein assay dye Reagent Concentrate; Bio-Rad laboratories, Solna, Sweden). Finally, in order to exclude any possible interactions between the chemical reagents and lucigenin, appropriate blank samples were analysed (i.e. PBS with febuxostat 30 nM and NDBP 100 μM without tissue), and the signal from the same period of measurement was not detectable.

Autoxidation of pyrogallol. To evaluate a possible direct effect of NDBP on superoxide scavenging, autoxidation of pyrogallol was used as a source of superoxide. In brief, pyrogallol (24 mM in 10 mM HCl) was mixed with phosphate buffer (100 mM, pH 8.0), and either vehicle (PBS), ascorbic acid (0.1 or 1 mM) or NDBP (0.1 or 1 mM) were added (Marklund & Marklund, 1974). The kinetics of pyrogallol autoxidation were measured using a spectrophotometer (Spectramax Plus 384 UV/VIS, Molecular Devices Corporation, CA, USA) at 420 nm and 25°C for 5 min. AUC was calculated for each measurement and values are presented as % (vs. vehicle group = 100%).

Part II: in vivo studies using a model of renal hypertension

After baseline BP measurements, C57BL/6J mice (n = 24, 25–30 g) were divided into four groups: control, control + NDBP, ANG II and ANG II + NDBP (n = 6 per group). Osmotic minipumps with ANG II (400 $ng \cdot kg^{-1} \cdot min^{-1}$) were implanted for the induction of hypertension. All animals were treated with NDBP (40 $mg \cdot kg^{-1} \cdot day^{-1}$, i.p.) or vehicle (cremophor in saline) twice daily (at 08:00 and 20:00 h), for five days, and BP measurements were recorded before and during ANG II infusion, with or without NDBP treatment (Supporting Information Figure S1b). At termination, the animals were killed, and plasma and tissues samples were collected for *in vitro* analyses.

BP monitoring. BP was monitored using a noninvasive tail-cuff system (Kent Scientific Corporation Coda, CT, USA). Measurements were conducted following the manufacturer's protocol, and BPs were assessed during basal conditions, with and without ANG II infusion, before and after treatment with NDBP.

Implantation of osmotic minipumps. Mice were anaesthetized by subjecting them to spontaneous inhalation of isoflurane (Forene®, Abbott Scandinavia AB, Solna, Sweden) in air (~2.2%), and osmotic minipumps (Alzet®, Durect™, CA, USA) were implanted s.c., delivering ANG II (Sigma-Aldrich, Stockholm, Sweden) for 14 days, as described previously (Kawada *et al.*, 2002; Carlstrom *et al.*, 2010a; Gao *et al.*, 2011). In sham-operated animals, the same procedure was

conducted but without implantation of the osmotic minipump.

Termination and sample collection. The mice were anaesthetized with isoflurane (as described above) whereupon the abdomen was opened using a midline incision, and blood samples were collected from the inferior vena cava. The whole blood with 2 mM EDTA (Sigma-Aldrich) was centrifuged immediately at 4°C for 7 min (6000 *g*); plasma was aliquoted and frozen for later analyses [10 μM IBMX (non-selective PDE inhibitor) were added immediately for later cGMP measurement]. The heart and kidneys were explanted, blotted, and the organs were weighed. The transverse section of kidney including renal hilum and transverse section of heart including both left and right ventricles were washed in cold PBS and placed in a solution of methanol (80%) and DMSO (20%) at −80°C. After 5 days, the samples were dehydrated by washing steps in ethanol (75, 50 and 25%) and embedded in OCT (TissueTek, Sakura, Japan) for histology or detection of superoxide (described below). The remaining renal and cardiac tissues were snap frozen in dry ice and stored at −80°C until analysis.

Histological examination. Frozen kidneys and hearts were sectioned (5 μm thick) using cryostat and stained with picrosirius red. The renal and cardiac morphology/histology was evaluated in a blinded fashion, and the damage scores were calculated essentially as described previously (Sallstrom *et al.*, 2013). The tissues evaluated were given a score of 0–4 depending on the severity of change.

Plasma markers. Plasma samples containing IBMX (10 μM) were analysed using a cGMP ELISA kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Plasma nitrate and nitrite levels were analysed by HPLC (ENO-20) and auto-sampler (840, EiCom, Kyoto, Japan), as described previously (Carlstrom *et al.*, 2010b; Hezel *et al.*, 2015). The samples were extracted using methanol (1:2) and then centrifuged at 4°C for 10 min (10000 *g*). Nitrate and nitrite were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analysed by detection at 540 nm.

Fluorescent detection of superoxide with DHE. For the analysis of superoxide accumulation, the fluorescent superoxide indicator dihydroethidium (DHE) was used as previously described (Cheng *et al.*, 2008; Kuroda *et al.*, 2010). Cryosections (10 μm) were incubated with DHE (3 mM) overnight at 4°C in the dark. DHE permeates cell membranes, and upon reaction with superoxide, it turns into the red fluorescent ethidium. Ethidium fluorescence

Table 1

Sequences of primers used for qPCR

Gene	Primer	Sequence (5′–3′)
Nox2	m_NOX2-fw	GCACCTGCAGCCTGCCTGAATT
	m_NOX2-rev	TTGTGTGGATGGCGGTGTGCA
Nox4	m_NOX4-fw	GGCTGGCCAACGAAGGGGTTAA
	m_NOX4-rev	GAGGCTGCAGTTGAGGTTCAGGACA
p22phox	m_p22-fw	CTGGCGTCTGGCCTGATTCTCATC
	m_p22-rev	CCGAAAAGCTTCACCACAGAGGTCA
p47phox	m_p47-fw	CAGCCATGGGGGACACCTTCATT
	m_p47-rev	GCCTCAATGGGGAACATCTCCTTCA
p67phox	m_p67-fw	AAGACCTTAAAGAGGCCTTGACGCA
	m_p67-rev	TCGGACTTCATGTTGGTTGCCAA
XO	m_XO-fw	CAGCATCCCCATTGAGTTCA
	m_XO-rev	GCATAGATGGCCCTCTTGTTG
Actin	m_actin-fw	GCTCCTCCTGAGCGCAAT
	m_actin-rev	GTGGACAGTGAGGCCAGGAT

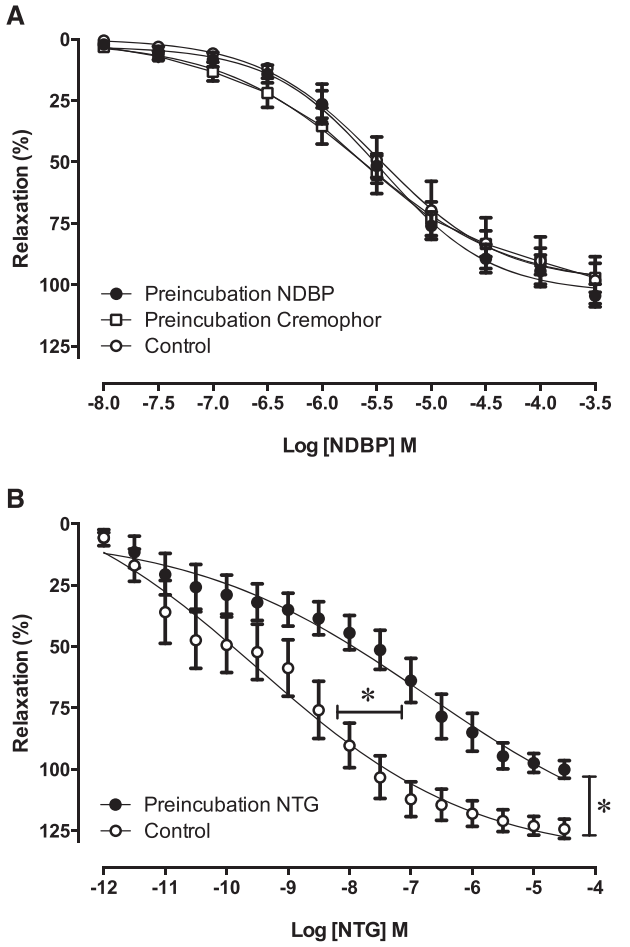

Figure 1

Vascular reactivity. Vascular relaxation studies in mesenteric artery, using a wire-myograph system to assess tolerance for NDBP and NTG. Previous exposure to NDBP (100 μM; 60 min) did not change the relaxation to cumulative doses of NDBP (10^{-8} to 10^{-4} M) (A). The cremophor alone did not induce any effects on the artery rings pre-contracted with PE. In contrast, pre-incubation with nitroglycerin (NTG, 100 μM), at the same concentration as used for NDBP, significantly attenuated NTG-mediated vasodilatation (B). Data are shown as mean ± SEM (n = 6 per group). *$P < 0.05$.

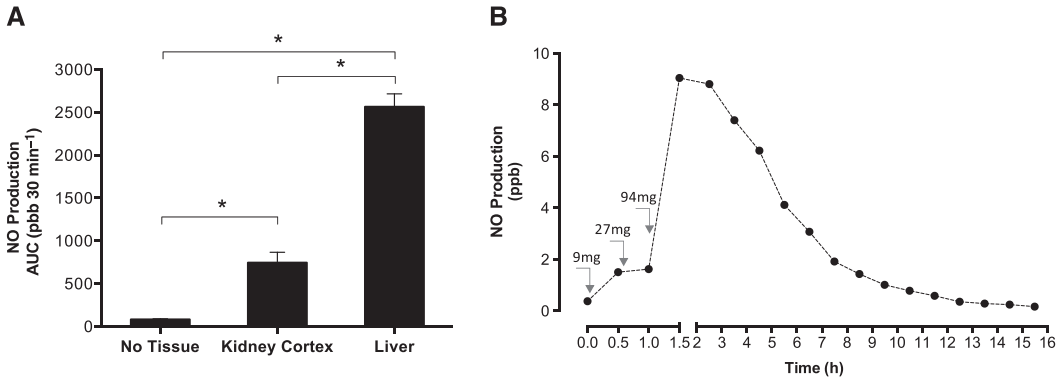

Figure 2

NO production. (A) Effect of NDBP (3.6 mM) on NO production (ppb) in a cell-free system and in the presence of the renal cortex and liver homogenates. Data are shown as mean ± SEM ($n = 5$ per group). *$P < 0.05$. (B) *In vitro* characterization of the NO responses to different doses of NDBP (9, 27 and 94 mg) in the presence of liver homogenate (equal to 3.6, 10 and 37 mM).

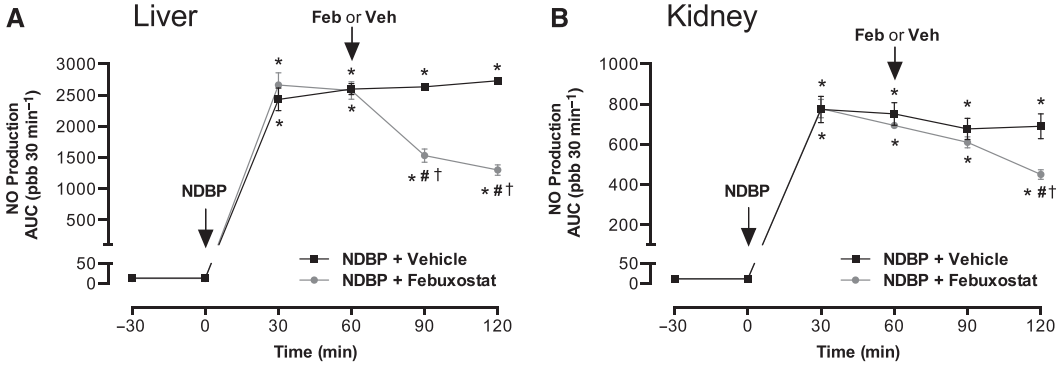

Figure 3

Effects of xanthine oxidase inhibition on NO production. Inhibition of xanthine oxidase with febuxostat (1 μM) in liver (A) and renal cortex (B) homogenates pre-incubated with NDBP (18 mg; equal to 6.85 mM). Data are shown as mean ± SEM (n = 5 per group). *$P < 0.05$ versus pre-incubation with NDBP. #$P < 0.05$ versus 30 and 60 min, †$P < 0.05$ versus NDBP + vehicle.

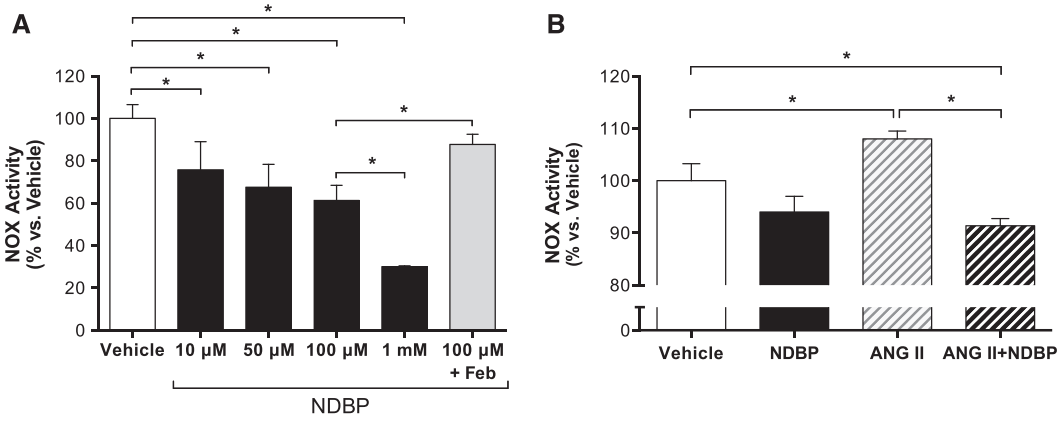

Figure 4

NADPH oxidase activity. Effect of NDBP on NOX activity in liver (A) and renal cortex (B) homogenates. (A) Dose-dependent response to NDBP in liver and during simultaneous inhibition of xanthine oxidase with febuxostat (Feb; 30 nM) ($n = 5$ per group). (B) Effect of 100 μM NDBP on NOX activity in renal cortex homogenates with and without pre-incubation with angiotensin II (ANG II; 1 μM). Data are shown as mean ± SEM ($n = 5$ per group). *$P < 0.05$.

was captured using a Zeiss Axiophot fluorescence microscope (Zeiss Axiovert 200 M, software Axiovision 3.0). For each tissue section, DHE fluorescence was measured in five to eight representative areas and calculated from 10 separate high power fields in each image using image J software.

NOX activity. The kidney cortex from control, ANG II and ANG II + NDBP treated mice was homogenized and prepared for NOX activity, using lucigenin-dependent chemiluminescence technique as described above.

qPCR. Total RNA was isolated from kidney using the RNeasy Mini Kit (Qiagen, Sollentuna, Sweden). In brief, 10–15 mg of kidney cortex were homogenized in RLT buffer (RNeasy Mini Kit) by adding zirconium oxide beads (0.5 mm) using the bullet blender (Next Advance, Inc.). After centrifugation, the homogenate was mixed with an equal volume of 70% EtOH, and total RNA was isolated according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with the High Capacity Reverse Transcription kit (Life Technologies, Stockholm, Sweden). The quantitative real-time PCR (qPCR) was performed in duplicates on an ABI 7500 Real-Time PCR System using 8.3 ng cDNA, 1× Power SYBR Green PCR Master Mix (Life Technologies) and 5 pmol of respective gene specific primers (Table 1). Expression levels were normalized to β-actin using the ΔΔCt method and are expressed as fold change versus controls.

Ethics. The experiments were approved by the ethics committee in Stockholm (Sweden) for animal experiments, or Animal Use and Care Committee at Federal University of Paraiba in João Pessoa (Brazil), and were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Data analysis. The *in vivo* and *in vitro* experiments were conducted in a double- or semi-blinded fashion, and the treatment protocols or groups were randomized. Data are presented as means ± SEM. Two-way repeated-measures ANOVA was used to test time- or concentration-dependent changes and to assess differences between the groups (vascular reactivity, NO production and NOX activity). *Post hoc* comparisons were performed with the Holm-Sidak's test. Ordinary one- or two-way ANOVA, followed by the Holm-Sidak's test, when appropriate, was used for other comparisons among groups. Statistical significance was defined as $P < 0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

Tolerance test for NDBP in mesenteric artery

In PE pre-contracted mesenteric artery rings isolated from normotensive rats, cumulative administration of NDBP (10^{-8} to $10^{-3.5}$ M) produced a concentration-dependent and endothelium-independent vasorelaxation. Previous exposure to a high concentration of NDBP (100 μM) for 60 min did not change the NDBP-induced effect (Figure 1A). The cumulative addition of cremophor (10^{-8} to $10^{-3.5}$ M) did not induce any effect on the artery rings pre-contracted with PE. Moreover, previous exposure to cremophor (100 μM) did not affect NDBP-induced relaxation (Supporting Information Figure S2). Of note, previous incubation (60 min) with the classic organic nitrate, NTG (100 μM), attenuated vasodilatation induced by cumulative addition of NTG compared with application under control conditions (Figure 1B). These data

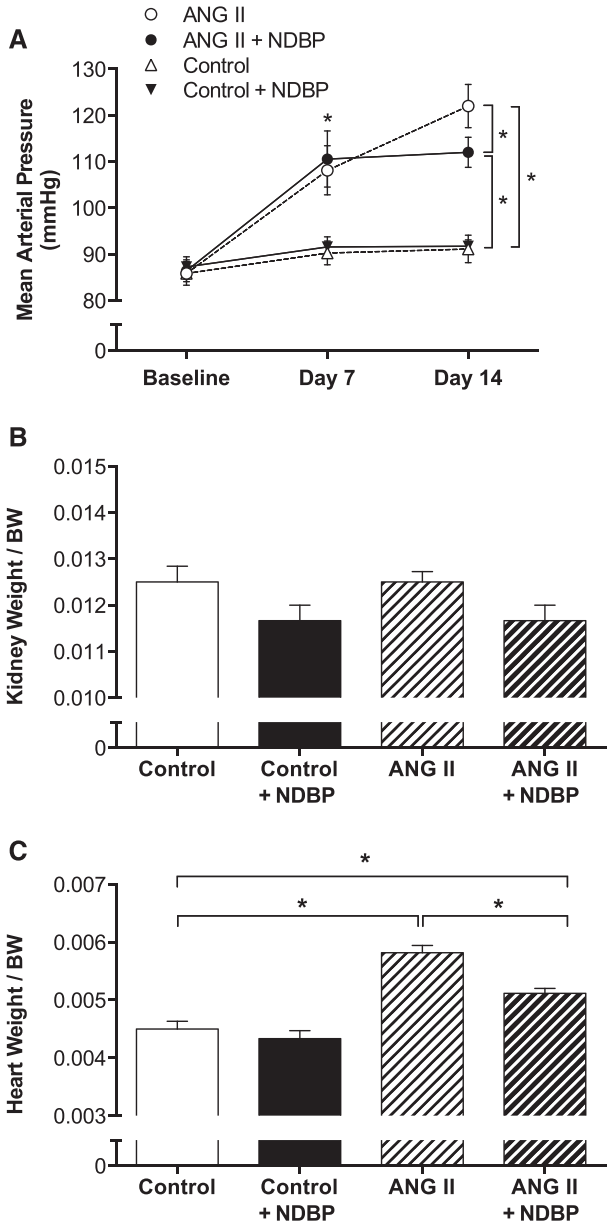

Figure 5

Progression of hypertension. Effects of chronic treatment with NDBP in an *in vivo* model of ANG II-induced hypertension. Mean arterial pressure (A), relative kidney weight (B) and heart weight (C). Data are shown as mean ± SEM ($n = 6$ per group). *$p < 0.05$ between indicated groups or compared with Baseline (A). BW; body weight.

suggest that NDBP, in contrast to NTG, does not cause tolerance *in vitro*.

Effect of NDBP on NO production

Administration of NDBP slightly increased NO production in the cell-free system compared with vehicle (Figure 2A). In the presence of tissue homogenates, this effect on NO production was strongly potentiated, with almost 10-fold and 30-fold higher NO production in the kidney and liver respectively. These results with NDBP suggested both non-enzymatic and enzymatic mechanisms for NO release; however, further mechanistic studies are needed to confirm this idea. Incubation with NTG also increased NO production in the liver (Supporting Information Figure S3a), but to a lesser degree compared with NDBP. Increasing doses of NDBP further enhanced NO production in the liver (Figure 2B). After using a 10-fold higher dose of NDBP, compared with that used in Figure 2A, NO production was sustained for more than 12 h.

Inhibition of XO with febuxostat significantly reduced NDBP-mediated NO production in liver and renal cortex compared with vehicle (Figure 3A and B). This effect was observed in both organs, although more pronounced in the liver.

Effect of NDBP on NOX activity

Incubation with NDBP reduced NOX-mediated superoxide production in liver homogenate. This effect was dose-dependent, and simultaneous administration of the XO inhibitor febuxostat abolished the effect of NDBP on NOX activity (Figure 4A). NTG also reduced liver NOX activity dose-dependently, but only at higher concentrations (Supporting Information Figure S3b).

In renal cortex, incubation with NDBP (100 μM) alone did not significantly reduce NOX activity (P= 0.09). As demonstrated previously (Gao *et al.*, 2015), treatment with ANG II increased NOX activity, and this activation was completely inhibited in the presence of NDBP (Figure 4B).

Effect of NDBP on BP during chronic ANG II infusion

Chronic infusion with a slow-pressor dose of ANG II is a well established animal model of renal hypertension (Kawada *et al.*, 2002; Crowley *et al.*, 2005; Crowley *et al.*, 2006). One week after implantation of osmotic minipumps in mice, both groups with ANG II infusion displayed similar degree of BP elevation (Δ: 22 ± 6 and 24 ± 6 mmHg), compared with baseline. In sham-operated control mice, BP was not significantly

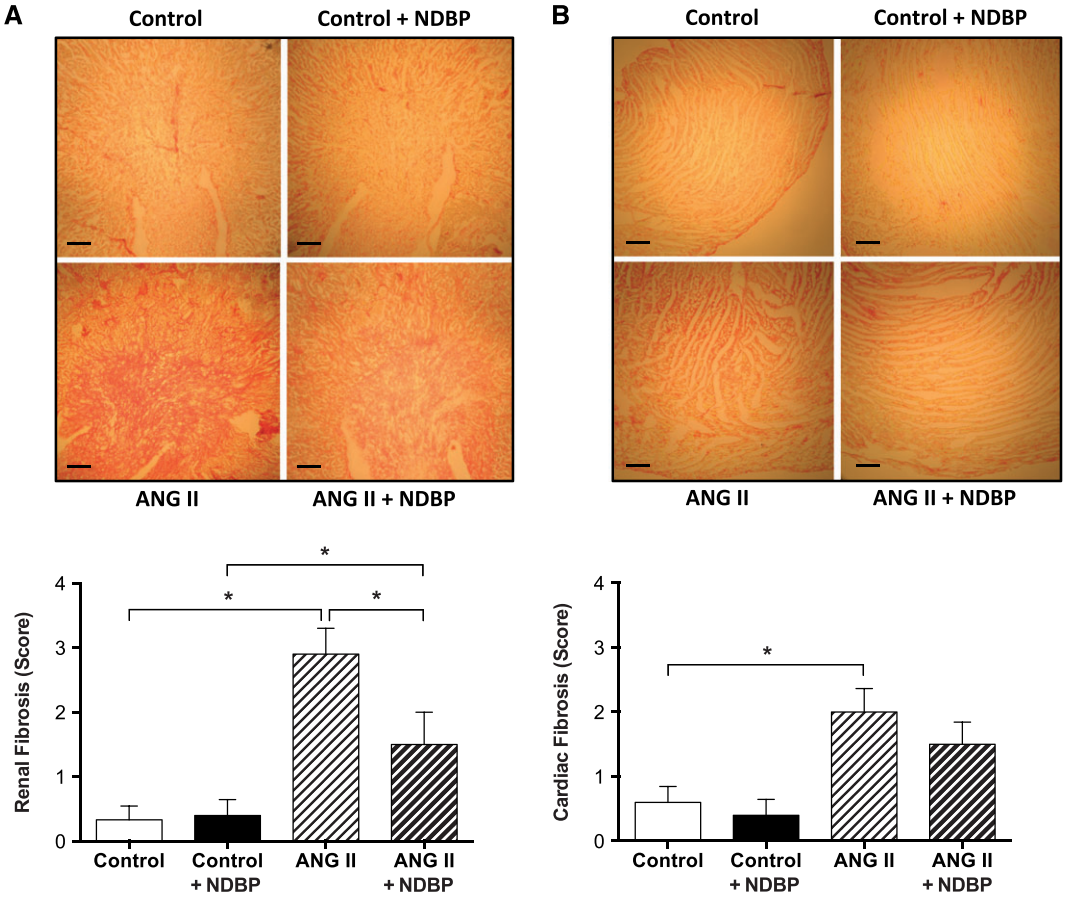

Figure 6

Renal and cardiac fibrosis. Effects of chronic treatment with NDBP on renal and cardiac fibrosis in mice with ANG II-induced hypertension. Representative kidney (A) and heart (B) sections together with calculated damage scores from the experimental groups. Data are shown as mean ± SEM (n = 6 per group). *P < 0.05.

changed, although there was a trend for slightly higher BP in both groups (Δ: 4 ± 2 mmHg, p = 0.06). Between day 7 and 14, hypertension was progressing in the ANG II group (Δ: 14 ± 6 mmHg, $P < 0.05$), whereas no change in BP was observed in the ANG II infused mice with simultaneous NDBP treatment during this period (Δ: 1 ± 5 mmHg). Importantly, in sham-operated mice without ANG II infusion, no effect on BP was observed with NDBP treatment (Figure 5A & Supporting Information Figure S4). The cremophor *per se* had no significant effect on BP and heart rate (Supporting Information Figure S5).

ANG II-induced hypertension was not associated with any changes in kidney weight (Figure 5B), but heart weight was increased in both groups (Figure 5C). Cardiac hypertrophy was significantly attenuated in ANG II infused mice with NDBP treatment, whereas no effect of NDBP alone was observed in control mice.

Effect of NDBP on renal and cardiac histology following chronic infusion with ANG II

ANG II-induced hypertension was associated with fibrotic changes of the kidney, which were primarily located in the corticomedullary region (Figure 6A). Simultaneous treatment with NDBP significantly attenuated the degree of fibrosis. Moreover, cardiac hypertrophic changes in ANG II-infused mice were associated with focal fibrosis, mainly located in the left ventricular wall (Figure 6B). The degree of fibrosis was less pronounced compared with the kidneys, and the damage score was only found to be significantly increased in the group with ANG II infusion alone.

Effect of NDBP on circulating nitrate, nitrite and cGMP levels following chronic infusion of ANG II

Plasma levels of both nitrate and nitrite were significantly elevated by NDBP in control mice, whereas cGMP levels were not affected (Figure 7A–C). Infusion with ANG II did not significantly change nitrate/nitrite levels, although plasma nitrate clearly tended to increase. In conjunction with this, a trend for reduced cGMP was noted. ANG II-treated mice with NDBP displayed higher nitrate and nitrite levels, whereas no significant increase in cGMP was observed compared with ANG II treatment alone.

Effect of chronic infusion of ANG II on XO

Our *in vitro* data suggested that XO may be involved in NDBP-mediated NO production. Chronic infusion of ANG II in mice resulted both in significantly elevated kidney XO mRNA and plasma uric acid levels (indicative of XO activity), and this was not changed by simultaneous treatment with NDBP (Supporting Information Figure S6a–b).

Effect of NDBP on oxidative stress following chronic infusion of ANG II

Several studies have previously demonstrated that systemic infusion with a low dose of ANG II is associated with increased oxidative stress in rats and mice (Kawada *et al.*, 2002; Carlstrom *et al.*, 2009; Carlstrom *et al.*, 2010a; Gao *et al.*, 2011; Gao *et al.*, 2015). To further understand the effect of NDBP in this model of hypertension, we measured superoxide production using the fluorescent superoxide indicator

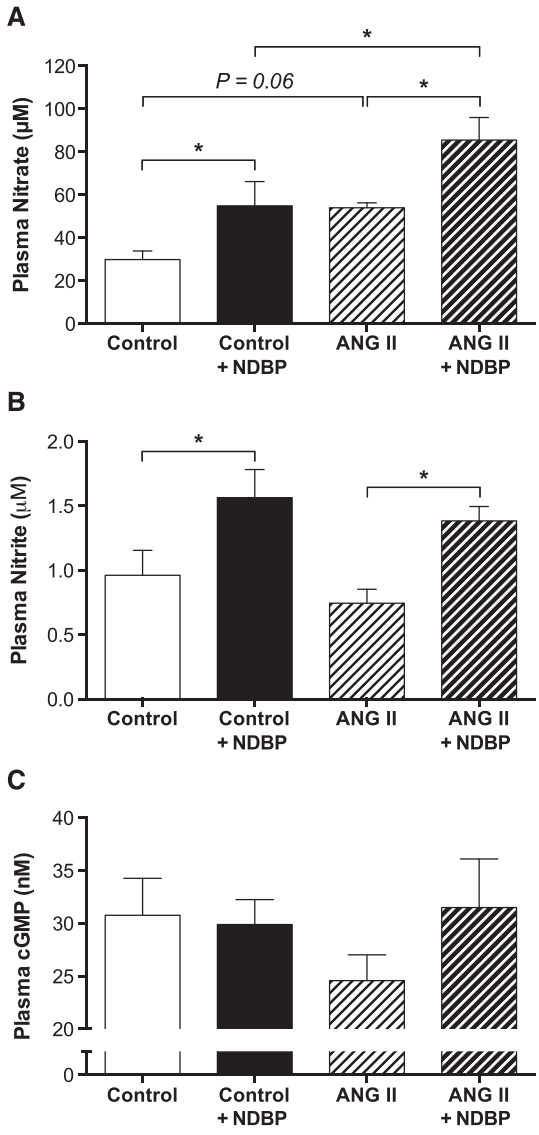

Figure 7

Markers of NO signalling. Effects of chronic treatment with NDBP on plasma nitrate (A), nitrite (B) and cGMP (C) levels in an *in vivo* model of ANG II-induced hypertension. Data are shown as mean ± SEM (n = 6 per group). *$P < 0.05$.

DHE. Infusion of ANG II was associated with increased DHE staining in both kidney and heart tissues, whereas this was not changed in ANG II-treated mice with NDBP (Figure 8A and B). As expected, NOX activity was increased in the kidney of ANG II-infused mice compared with controls (198 ± 38 vs. 100 ± 13%). However, this was attenuated in mice simultaneously treated with NDBP (Supporting Information Figure S7a). Surprisingly, NOX activity was not significantly increased in the heart of mice with ANG II compared with controls (124 ± 25 vs. 100 ± 10%), and this was not changed by NDBP treatment.

To further investigate the mechanism(s) of NDBP-mediated reduction in renal NOX activity following infusion of ANG II, mRNA expression studies were conducted (Supporting Information Figure S7b–f). Among the isoforms and subunits

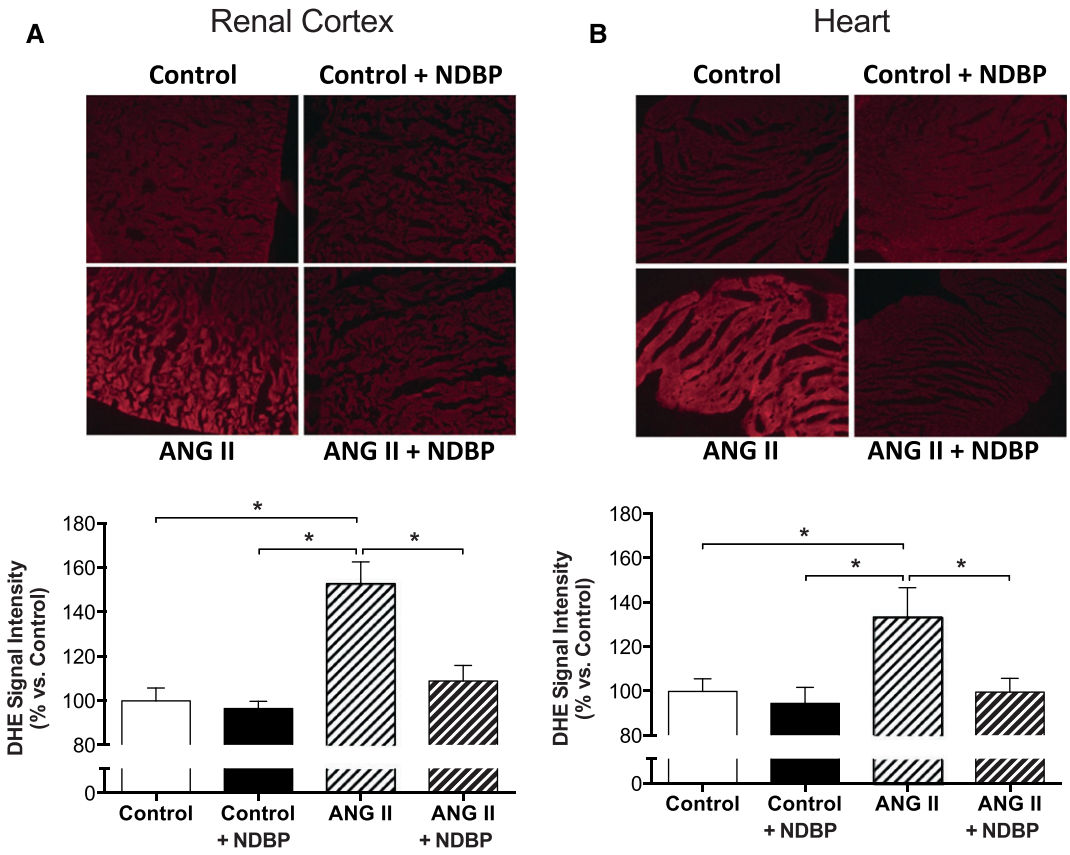

Figure 8

Renal and cardiac oxidative stress. Effect of chronic NDBP treatment on superoxide accumulation in renal cortex (A) and heart (B) from mice with ANG II infusion. Representative microphotographs of tissue sections with DHE-driven fluorescence used for determining superoxide accumulation are shown in the top panels. Data are shown as mean ± SEM ($n = 6$ per group), $^*P < 0.05$.

investigated, p22phox and p67phox were increased in mice after ANG II infusion. However, this was not prevented in mice with co-treatment of NDBP. Finally, to investigate if the NDBP compound could act directly as an antioxidant we carried out autoxidation studies of pyrogallol. Superoxide levels were reduced with the antioxidant ascorbic acid; however, incubation with NDBP did not alter superoxide formation (Supporting Information Figure S8). Taken together, these experiments suggest NDBP has a modulatory effect on the NOX function, rather than inducing the down regulation of NOX expression or direct scavenging of superoxide by NO.

Discussion and conclusions

In the battle against cardiovascular disease, and its adverse complications, new strategies to increase NO production or bioavailability have gained a lot of interest (Lundberg *et al.*, 2015). The present study provides evidence that the newly discovered and synthesized organic nitrate NDBP not only generates NO in the acute setting but also that chronic treatment can attenuate the development of ANG II-induced hypertension and associated organ pathologies. Moreover, our vascular data indicate that treatment with NDBP, in contrast to treatment with the classic organic nitrate NTG, is not associated with tolerance. In addition, the effects of NDBP on both NO release and NOX activity were more pronounced compared with those of NTG. Although the underlying mechanisms require further attention, our studies clearly suggest that inhibition of superoxide production in the kidney is an important component.

Previous studies using NDBP showed that acute treatment induces vasorelaxation and BP reduction through activation of the NO-sGC/cGMP pathway and also modulation of K^+ channels (Franca-Silva *et al.*, 2012a; Franca-Silva *et al.*, 2012b). Considering the undesirable effects with existing organic nitrates, especially with frequent dosing, additional studies to advance the understanding of the effects elicited by NDBP have been warranted. Here, we used a well-established model of hypertension, in which BP progressively increases during chronic infusion of ANG II. This model has been proven to be of renal origin (Crowley *et al.*, 2005; Crowley *et al.*, 2006), and stimulation of NOX-derived oxidative stress is a crucial component in the pathogenesis (Kawada *et al.*, 2002; Landmesser *et al.*, 2002; Carlstrom *et al.*, 2009; Lai *et al.*, 2012). Previous studies in mice have demonstrated that simultaneous administration of superoxide scavenger tempol (Elmarakby *et al.*, 2007), inhibition of NOX with apocynin (Li *et al.*, 2013) and genetic disruption of the NOX isoform (Matsuno *et al.*, 2005; Carlstrom *et al.*,

2009) or its cytosolic subunits (Landmesser *et al.*, 2002; Lai *et al.*, 2012) attenuate ANG II-induced hypertension. Here, we showed that NDBP treatment in mice, which already displayed elevated BP, prevented further increase in BP during the remaining period with ANG II infusion. This favourable effect of NDBP was also associated with less fibrosis in both renal and cardiac tissues. In contrast to that observed in studies with acute NDBP treatment, our *in vivo* studies with chronic NDBP treatment failed to detect any significant increase in plasma cGMP. However, successful detection of increases in this second messenger depends on many factors (e.g. timing of dosing, short vs. long-term treatment, mode of administration and basal cGMP levels). Similar to what has been proposed in some studies using inorganic nitrate or nitrite, it is possible that NDBP-mediated effects to some extent are cGMP-independent (e.g. via formation of nitros(yl)ation products), which has been proposed for nitrate-mediated vascular (Carlstrom *et al.*, 2015; Liu *et al.*, 2015) and metabolic effects (Carlstrom *et al.*, 2010b).

Developing new organic nitrates devoid of side effects may represent an attractive strategy to find new clinical applications for this class of drugs. For example, the nitrate nicorandil has been associated with improved endothelial function and inhibition of coronary artery events while being devoid of clinical tolerance (Sekiya *et al.*, 2005). The mononitrate aminoethyl nitrate showed an almost similar potency compared with NTG; however, it induced severe *in vivo* tolerance (Schuhmacher *et al.*, 2009). Amongst others, these nitrates as well as our novel compound NDBP share the actions of nitrates and K^+ channel activators, suggesting great potential for this class of nitrate hybrid molecules in prevention and treatment of hypertension and associated cardiovascular diseases. However, further characterization of the bioactivation, tolerance and mechanism of action are needed for each nitrate since there are major differences between different molecules.

Many of the biological effects of both organic and inorganic nitrates are generally believed to involve the release of NO from nitrite followed by activation of GC (Nossaman *et al.*, 2010). Bioactivation of organic nitrates (e.g. NTG, isosorbide nitrates) requires thiols or sulfhydryl containing compounds, and more recent studies demonstrated that an enzymatic mechanism, such as mitochondrial aldehyde dehydrogenase and P450 enzymes, catalyzes the formation of glyceryl dinitrate and nitrite from NTG (Chen *et al.*, 2002; Ignarro, 2002). Regarding inorganic nitrite, a number of proteins and enzymes have been implicated in the reduction step to NO or NO-like compounds, including deoxygenated haemoglobin, myoglobin, mitochondrial respiratory chain enzymes, aldehyde oxidase and in particular XO (Lundberg *et al.*, 2009; Castiglione *et al.*, 2012). The crucial involvement of aldehyde dehydrogenase has been proven (Chen *et al.*, 2002), but less is known about the specific role of XO in the bioactivation of organic nitrates. However, recent studies have demonstrated that vascular relaxation to inorganic nitrite, but not NTG, were attenuated by allopurinol (Golwala *et al.*, 2009; Nossaman *et al.*, 2010; Azarmi *et al.*, 2014). On the contrary, Azarmi and colleagues concluded that inhibition of XO could reverse NTG-induced tolerance in rat thoracic aorta (Azarmi *et al.*, 2014). Interestingly, our findings suggest that NDBP-mediated NO production and reduction of NOX activity required functional XO, thus suggesting a potential difference between our compound and existing organic nitrates such as NTG. In addition, we observed increased XO expression and activity during ANG II infusion in mice both with and without simultaneous NDBP treatment, which supports the involvement of XO and could partially explain the protective effects of NDBP during ANG II infusion.

We have recently demonstrated that dietary supplementation with inorganic nitrate, or acute treatment with sodium nitrite, attenuates oxidative stress, inflammation and hypertension in models of renal and cardiovascular disease (Carlstrom *et al.*, 2011; Gao *et al.*, 2015; Peleli *et al.*, 2015; Yang *et al.*, 2015). Regarding the effects observed with NDBP in this study, a remaining question that warrants further investigations is the exact mechanism for NDBP-mediated reduction in NOX activity. Antioxidant properties by NDBP or direct scavenging of superoxide by NDBP-mediated NO is one possibility, although this was not supported by our pyrogallol autoxidation results in this study. A reduction of NOX expression by NDBP would represent a second alternative, although our mRNA expression data of NOX and its subunits did not give strong support for this. A third, and more likely mechanism is the direct inhibition of NOX activity by NO or reactive nitrogen oxides, for example through nitrosation of critical thiols in the catalytic site of the enzyme or via nitration reactions (Selemidis *et al.*, 2007; Wolin, 2009; Yun *et al.*, 2011; Qian *et al.*, 2012). Future studies are warranted to investigate this hypothesis.

In conclusion, we report for the first time that repeated treatment with the new organic nitrate NDBP has favourable effects in a model of renal hypertension associated with oxidative stress. Increased NOX-derived superoxide formation has a central role in the pathogenesis of several cardiovascular disorders, including hypertension, and antioxidant therapy has been suggested as a therapeutic approach (Drummond *et al.*, 2011). However, results with existing antioxidants have been rather disappointing and may be explained by their relatively low effectiveness compared with compounds used in experimental studies (Wilcox, 2010). Treatment with an ANG II receptor blocker has proven to lower oxidative stress and increase NO bioavailability (Nguyen Dinh Cat *et al.*, 2013). We observed a robust reduction in NOX activity with NDBP in our study, which was associated with attenuated BP elevation in a model of hypertension. Interestingly, similar effects on both NOX activity and ANG II-induced hypertension have been observed with inorganic nitrate and nitrite. Therefore the mechanism of action of NDBP could possibly be dependent on metabolism, or release of nitrite from the molecule, which is then reduced by XO to NO, rather than rapid metabolism to NO with subsequent oxidation to nitrite. However, future studies are warranted to elucidate the mechanisms and also compare its effects with existing antihypertensive drugs and with inorganic nitrate/nitrite. Taken together, our *in vivo* and *in vitro* findings suggest absence of tolerance, and a novel mechanism where NDBP-mediated NO release inhibits NOX-derived oxidative stress. We believe that results from this study will help to advance the field towards clinical trials, considering that currently used organic nitrates exhibit several undesirable effects with frequent dosing.

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Author contributions

Conception and design were carried out by S.K.P.P., C.Z., M. P., M.F.M., M.C. Development of methodology was carried out S.K.P.P., C.Z., M.P., M.F.M., M.C.R.B., P.F.A.F., M.S.F.S., M.C. Acquisition of data was carried out by S.K.P.P., C.Z., M. P., M.F.M., M.C.R.B., V.A.B., M.C. Analysis and interpretation of data were carried out by S.K.P.P., C.Z., M.P., M.F.M., E.L., V.A.B., M.C. Writing study supervision was carried out by J.O.L., E.W., E.G.P., V.A.B., M.C.

Conflict of interest

The authors declare no conflicts of interest.

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 recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13511

Figure S1 Design of the *in vitro* (A) and *in vivo* (B) protocols. NDBP, 2-nitrate-1,3-dibuthoxypropan; NTG, nitroglycerin, NOX, NADPH oxidase; NO, nitric oxide; ANG II, angiotensin II; i.p, intraperitoneal; DHE, dihydroethidium; XO, xanthine oxidase.
Figure S2 *In vitro* studies in mesenteric arteries to assess the effects of the cremophor. Increasing concentrations of the cremophor alone (10^{-8} to 3 x 10^{-4} mol/L) had no significant

effects on vascular relaxtion, and simultaneous incubation with a high dose of the cremophor (100 μmol/L) did not influence NDBP-mediated vascular relaxation. Data are shown as mean±SEM (n = 6/group).

Figure S3 Effect of NTG (3.6 mM) on nitric oxide production (ppb) in a cell-free system and in the presence of liver homogenates (n = 5/group) (A). Effect of increasing doses of NTG on NOX activity in liver homogenates (Vehicle, n = 14; 10 μM, n = 6; 50 μM, n = 6; 0.1 mM, n = 8; 0.5 mM, n = 6; 1 mM, n = 14; 3.6 mM, n = 6) (B). Data are shown as mean ±SEM. *$P < 0.05$.

Figure S4 Effects of chronic treatment with NDBP in an *in vivo* model of ANG IIinduced hypertension. Systolic (A) and diastolic (B) BP are shown as mean±SEM (n = 6/group). *$P < 0.05$ between indicated groups or compared with Baseline.

Figure S5 *In vivo* studies to assess the effects of the cremophore on cardiovascular function in anaesthetized rats. Original recordings of pulse arterial pressure (PAP), mean arterial pressure (MAP), and heart rate (HR) are shown in panel A (traces for each dose represent one min). Increasing doses of the cremophor had no effects on MAP (B) or HR (C). Data are shown as mean±SEM (n = 6/group).

Figure S6 Effect of chronic NDBP treatment on XO mRNA expression in renal cortex (A) and uric acid levels in plasma (B) from mice with ANG II infusion. Data are shown as mean ±SEM (n = 5/group). *$P < 0.05$.

Figure S7 Effect of chronic NDBP treatment on NOX activity (A) and expression of NOX isoforms and its subunits (B-F) in renal cortex from mice with ANG II infusion. Data are shown as mean±SEM (n = 6/group). *$P < 0.05$.

Figure S8 Assessment of superoxide scavenging capacity of NDBP compared with the antioxidant ascorbic acid, measured via their effect on pyrogallol autoxidation. Data are shown as mean±SEM (n = 6/group). *$P < 0.05$.