



## The novel organic mononitrate NDHP attenuates hypertension and endothelial dysfunction in hypertensive rats



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### ABSTRACT

**Rationale:** Development and progression of cardiovascular diseases, including hypertension, are often associated with impaired nitric oxide synthase (NOS) function and nitric oxide (NO) deficiency. Current treatment strategies to restore NO bioavailability with organic nitrates are hampered by undesirable side effects and development of tolerance. In this study, we evaluated NO release capability and cardiovascular effects of the newly synthesized organic nitrate 1, 3-bis (hexyloxy) propan-2-yl nitrate (NDHP).

**Methods:** A combination of *in vitro* and *in vivo* approaches was utilized to assess acute effects of NDHP on NO release, vascular reactivity and blood pressure. The therapeutic value of chronic NDHP treatment was assessed in an experimental model of angiotensin II-induced hypertension in combination with NOS inhibition.

**Results:** NDHP mediates NO formation in both cell-free system and small resistance arteries, a process which is catalyzed by xanthine oxidoreductase. NDHP-induced vasorelaxation is endothelium independent and mediated by NO release and modulation of potassium channels. Reduction of blood pressure following acute intravenous infusion of NDHP was more pronounced in hypertensive rats (two-kidney-one-clip model) than in normotensive sham-operated rats. Toxicological tests did not reveal any harmful effects following treatment with high doses of NDHP. Finally, chronic treatment with NDHP significantly attenuated the development of hypertension and endothelial dysfunction in rats with chronic NOS inhibition and angiotensin II infusion.

**Conclusion:** Acute treatment with the novel organic nitrate NDHP increases NO formation, which is associated with vasorelaxation and a significant reduction of blood pressure in hypertensive animals. Chronic NDHP treatment attenuates the progression of hypertension and endothelial dysfunction, suggesting a potential for therapeutic applications in cardiovascular disease.

### 1. Introduction

Arterial hypertension is a chronic degenerative disease of multifactorial etiology that involves functional alterations of the vascular, renal and central nervous systems [1–3]. Of note, hypertension is closely related to endothelial dysfunction mainly attributed to reduced nitric oxide (NO) bioavailability in the vascular wall [4,5] and increased oxidative stress, resulting in chronic and abnormal increase in vascular resistance [6].

NO is considered as the main endothelium-derived relaxing factor

regulating arterial blood pressure in several ways, including relaxation of vascular smooth muscle cells, inhibition of platelet aggregation, smooth muscle cell replication and neuronal communication [7–10]. This lipid soluble gas is synthesized from L-arginine by the action of NO synthases (NOS) [11,12]. NO exerts its biological action on the vascular smooth muscle by activating soluble guanylate cyclase (sGC), leading to increased intracellular cyclic guanosine monophosphate (cGMP) levels, which in turn activates protein kinase G (PKG), thus mediating vasodilation [13]. In addition to modulating the sGC-cGMP-PKG pathway, NO may also directly activate K<sup>+</sup> channels; including calcium-

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dependent  $K^+$  channels, whose action promotes vasorelaxation [5,14,15].

Thus, the search for new therapies which increase NO bioavailability in cardiovascular diseases (CVDs) has been the target of numerous studies. Of note, organic nitrates such as sodium nitroprusside and glyceryl trinitrate, which mimic the role of endogenous NO in biological systems, have been used in the treatment of several cardiovascular diseases; such as arterial hypertension and coronary artery disease [16–22]. Despite their beneficial effects in patients with cardiovascular disease, these drugs have several clinical limitations. These include short duration (half-life), high reactivity, low tissue selectivity and development of tolerance (reducing efficacy) with frequent dosing [23–26]. Moreover, side-effects including headaches (cerebral vasodilation), flushing, hypotension and tachycardia, which augments angina via increasing oxygen demand [26].

In order to obtain a novel NO donor with reduced detrimental effects, 1,3-bis (hexyloxy) propan-2-yl nitrate (NDHP) obtained from glycerin was recently synthesized [27]. The aims of the present study were to characterize this novel organic nitrate as an NO donor and vasodilator, and to investigate both the acute and chronic cardiovascular effects of NDHP treatment in experimental models of hypertension.

## 2. Materials and methods

### 2.1. Ethics

All experimental procedures were approved by the ethics committee in Stockholm, Sweden (Protocol ID: N139/15) for animal experiments, and by Federal University of Paraíba Animal Care and Use Committee (CEUA/UFPB, Protocol ID: 142/2015) in João Pessoa, Brazil. The experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### 2.2. Animals and surgical procedures

Male (250–300 g) and female (200–250 g) Wistar rats were kept in ordinary cages in a temperature-controlled room, 12:12 h light/dark cycle with standard rat chow (Labina®, Purina, Paulinea, SP, Brazil) and water *ad libitum*. Renovascular hypertension was induced by clamping the right renal artery (2K1C model) in rats as previously described [28]. Briefly, under combined ketamine and xylazine anesthesia (75 and 10 mg/kg, *i.p.*, respectively), a midline abdominal incision was made. The right renal artery was exposed and carefully separated from the right renal vein and surrounding tissues. A U-shaped silver clip (0.2 mm, internal diameter) was placed around the vessel, proximal to the abdominal aorta, and the wound was closed. Control sham operations, were performed in exactly the same way, with the exception of clipping the renal artery. After surgery, the rats were kept in their cages and remained untouched for six weeks in order to develop arterial hypertension.

### 2.3. Nitric oxide measurements by fluorescence microscopy

Indirect NO measurements were performed using fluorescence microscopy with the cell permeable 4,5-diaminofluorescein diacetate (DAF-2 DA) probe. Normotensive rats were euthanized and superior mesenteric artery rings (2–3 mm) were isolated and maintained in physiological Tyrode's 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), at 37 °C. Vascular rings were pre-incubated with DAF-2A (10  $\mu$ M; Calbiochem, San Diego, CA, USA), in darkness for 30 mins. Vascular rings were then fixed in Tissue-Tek® O.C.T.™ and frozen at –20 °C. Sections were cut (8  $\mu$ m) and collected using a CM 1900 cryostat (Leica Inc., Deerfield, IL, USA) and mounted on slides with coverslips. The sections were washed (PBS 0.1 M) and stimulated with: PBS 0.1 M;

NDHP (1 mM); NDHP (1 mM) + L-NG-nitroarginine (L-NNA, 100  $\mu$ M), an eNOS inhibitor; linsidomine (SIN-1, 10  $\mu$ M), an NO donor, SIN-1 (10  $\mu$ M) + L-NNA (100  $\mu$ M) for 30 min. To test whether NDHP signaling was mediated by xanthine oxidoreductase (XOR); another set of DAF-2 DA experiments was performed. After loading and washing (as described above), the sections were incubated with the xanthine oxidase inhibitor febuxostat (50  $\mu$ M, Sigma-Aldrich), or the combination of febuxostat (50  $\mu$ M) + NDHP (1 mM) for 30 min. After stimulation the sections were washed and processed as described above. 4',6-diamidino-2-phenylindole stain (DAPI) was used to visualize cell nuclei. The cytosolic NO levels were assessed by exciting DAF-2 DA at 480 nm using a xenon lamp and measuring the fluorescence at an excitation of 515–565 nm using a fluorescence microscope (Zeiss Observer Z1, Germany). The relative fluorescence intensity was calculated from images obtained using a digital camera (Axio Cam MRm, Zeiss, Germany) and ImageJ software 1.42q (Wayne Rasband, National Institutes of Health, USA). The NO content (A.U), represents the change in the fluorescence intensity, which is calculated as the difference between the final and basal fluorescence.

### 2.4. Assessment of NDHP-derived nitric oxide in purified XOR

NDHP-induced NO production was evaluated by using purified XOR enzyme. In brief, 2 mL of PBS containing XOR (0.05 U/mL, Roche, Cat. no: 10110434001), NADH (1 mM, Sigma, Cat. no: 10107735001) and increasing concentrations of NDHP (0.1, 1, and 1 mM) was added into a chamber of an Oxygraph-2k system (Oroboros instruments) under nitrogen atmosphere (oxygen  $\leq$  0.02%) at 37 °C. The oxygraph chamber was connected in line to a NO analyzer device (ECO Physics analyzer, CLD 77 A.M., Swiss), using nitrogen gas as carrier (400 mL/min). After a period of equilibration (approx. 5 min), NDHP was injected into the chamber and real-time NO production was recorded throughout the experiments, using a data acquisition system (AcqKnowledge v3.9, Biopac MP150), as described previously [27,29]. While no changes in NO formation were detected by NADH, XOR or NDHP alone, the combination of these three components rapidly induced NO production. NO production was recorded within 1 h post NDHP injection and, in a subset of experiments, the XOR inhibitor oxypurinol (100  $\mu$ M) was added 15 min before NDHP. The average NO signal (ppb) was calculated during 5 min periods at baseline and at maximum response to NDHP. NDHP-mediated NO release was calculated by subtracting the average NO values, after the addition of NDHP, from the NO values at baseline.

### 2.5. Vasorelaxant effect elicited by NDHP

After euthanasia, the superior mesenteric artery was isolated, placed in Tyrode's solution and adherent tissue was dissected. Vessels were cut into arterial rings (1–2 mm) which were continuously gassed with a carbogenic mixture (95%  $O_2$  and 5%  $CO_2$ ) and maintained at 37 °C, pH 7.4. Rings were mounted vertically on two  $\Delta$ -shaped stainless steel wires in a 10 mL tissue chamber connected to a tension transducer (PowerLab™, ADInstruments, MA, EUA). All rings were stabilized under 0.75 g resting tension for 1 h. The presence of functional endothelium was assessed by responses of rings to acetylcholine (ACh, 10  $\mu$ M), following phenylephrine stimulation (PHE, 10  $\mu$ M). Experiments were conducted in rings with and without endothelium. After preconstriction of mesenteric artery rings with PHE (1  $\mu$ M), cumulative doses of NDHP were added to the organ bath ( $10^{-12}$ – $10^{-3}$  M) in order to study the concentration-response. Data were expressed as maximum effect and potency ( $pD_2$ ). In some preparations, the rings were pre-incubated for 30 min with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 100  $\mu$ M), the NO scavengers 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO, 300  $\mu$ M) and hydroxocobalamin (HDX, 30  $\mu$ M), the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 mM). Additional

experiments investigated the effects of tetraethylammonium (TEA, 3 mM); a non-selective K<sup>+</sup> channel blocker, TEA (1 mM); a large conductance Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel blocker, glibenclamide (GLIB, 1 mM); a K<sub>ATP</sub> blocker, 4-aminopyridine (4-AP, 1 mM); a voltage-operated K<sup>+</sup> channel blocker and barium chloride (BaCl<sub>2</sub>, 30 μM); an inward-rectifier potassium channels (K<sub>IR</sub>) blocker. Inhibition was calculated by comparing the response elicited by NDHP in the absence and presence of inhibitors in the preparations. All drugs were obtained from Sigma-Aldrich (São Paulo-SP, Brazil). ODQ and GLIB were solubilized in DMSO and the remaining compounds were dissolved in distilled water. For functional studies using ODQ and GLIB, the final concentration of DMSO never exceeded 0.01% in the bath. This had no effect when tested in control preparations (data not shown).

## 2.6. Acute preclinical toxicity test for NDHP

Toxicity test was performed according to the "Guidelines for Testing of Chemicals" from The Organization for Economic Co-operation and Development [30]. Female rats (n = 3/group) were treated with single doses of 300 or 2000 mg/kg by oral administration of NDHP, while the control group received vehicle only [5% (v/v) Cremophor in saline]. For the behavioral changes and signs of toxicity, characteristic signs of central nervous system (CNS) or autonomic nervous system (ANS) activity were carefully observed every 15 min (0, 15, 30 and 60 min), after 4 h, and on daily basis for the following 14 days [31]. On day 15, the animals were euthanized and the organs (liver, heart, spleen, kidneys and lung) were harvested, weighed and examined macroscopically. The dose responsible for the death of 50% of the experimental animals (LD<sub>50</sub>) was estimated accordingly [30].

## 2.7. Acute cardiovascular effects of NDHP in normotensive and hypertensive rats

After six weeks, sham and 2K1C operated rats were anesthetized with ketamine and xylazine (75 and 10 mg/kg, i.p., respectively) and polyethylene cannulae were inserted into the inferior vena cava and abdominal aorta through vein and femoral artery for drug injections and arterial pressure recordings, respectively. Animals were placed in individual cages for 24 h with food and water *ad libitum*. Blood pressure measurements were recorded in conscious rats using a pressure transducer connected to an acquisition system (PowerLab; ADInstruments, Castle Hill, NSW, Australia) and LabChart 5.0 software (ADInstruments). NDHP was dissolved in saline and administered through venous catheter following baseline recordings (20 min). Blood pressure was evaluated before and after the administration of NDHP (1; 5; 10 and 20 mg/kg, i.v., randomly). The interval between the doses was 15 min, and the results were expressed as changes in mean arterial pressure (ΔMAP).

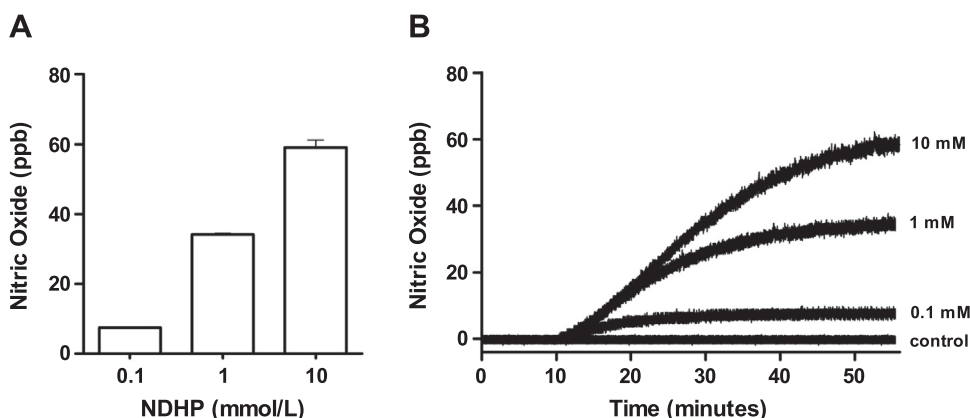


Fig. 1. Nitric oxide generation by NDHP. Effect of NDHP in different concentrations (0.1, 1 and 10 mM) on NO production (ppb) in a cell-free system. Data are shown as mean ± SEM. n = 3 per group.

## 2.8. Cardiovascular effects of repeated NDHP treatment in a rat model of angiotensin II-induced hypertension

Male Wistar rats (250–300 g, n = 16) from Janvier Labs (Saint Berthevin Cedex, France) were kept in ordinary cages in a temperature-controlled room, 12:12 h light/dark cycle with standard rat chow (Lantmännen, Kimstad, Sweden) and water *ad libitum*.

### 2.8.1. Cardiovascular function

Blood pressure and heart rate were monitored by noninvasive tail-cuff system (Kent Scientific Corporation Coda, Torrington, CT, USA), described previously [32,33] accordingly to the manufacturer's protocol. After training, baseline measurements were taken and subsequently the rats were anesthetized by spontaneous inhalation of isoflurane (Forene®, Abbott Scandinavia AB, Solna, Sweden) in air (~2.2%). Osmotic minipumps (Alzet®, Durect™, CA, USA) were implanted subcutaneously to deliver ANG II (120 ng/kg/min) (Sigma-Aldrich, Stockholm, Sweden) for 14 days, as described previously [34]. After implantation all rats were given L-NAME (0.5 g/L; drinking water) and treated with either NDHP (10 mg/kg i.p.) or vehicle (cremophor in saline) twice per day (8 a.m. and 8 p.m.). Blood pressure and heart rate were monitored again on Day 10–14 following implantation.

### 2.8.2. Endothelial function

On Day 15 the rats were euthanized; tissues samples were collected and snap frozen (−80 °C) for later *in vitro* analyses. Freshly isolated mesenteric arteries were mounted in multiwire myograph system for vessel reactivity studies [27,32].

### 2.8.3. Plasma nitrate and nitrite

Plasma samples were analyzed for their levels of nitrate and nitrite, using a dedicated high performance liquid chromatography (HPLC) system (ENO-20; Eicom) [27].

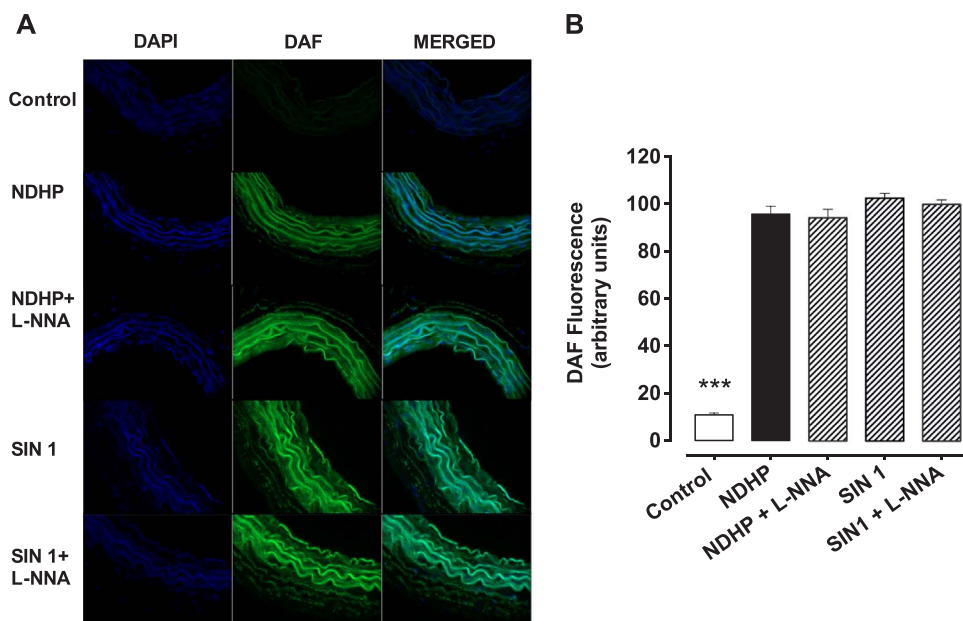
## 2.9. Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed by Student's *t*-test or analysis of variance (ANOVA; one or two two-way analysis as appropriate) followed by the recommended post-hoc analysis in the Graph Pad Prism 5.0 program. Statistical significance was considered when *p* < 0.05.

## 3. Results

### 3.1. NDHP-mediated nitric oxide generation in cell-free system – role of xanthine oxidoreductase

In the presence of NADPH and XOR, administration of NDHP (0.1, 1 and 10 mM) increased NO production in a dose-dependent manner



**Fig. 2.** Imaging of nitric oxide species in small arteries. (A) Representative images of fluorescence in superior mesenteric artery sections incubated with DAF-FM DA (10  $\mu$ M), a fluorescent cell permeable probe for NO. (B) Bars graph showing the NO production after incubation with only DAF-2DA (Baseline) and treatments groups: NDHP; NDHP + L-NNA; Linsidomine (SIN-1); SIN-1 + L-NNA. Fluorescence was quantified by optic densitometry (arbitrary units, a.u.). Data are shown as mean  $\pm$  SEM. n = 13–16 per group. \*p < 0.05 DAF versus other groups.

compared with control. NO production was sustained for more than 50 min (Fig. 1), and this effect was solely observed after the addition of XOR, thus suggesting that NDHP-mediated NO release requires enzymatic activity.

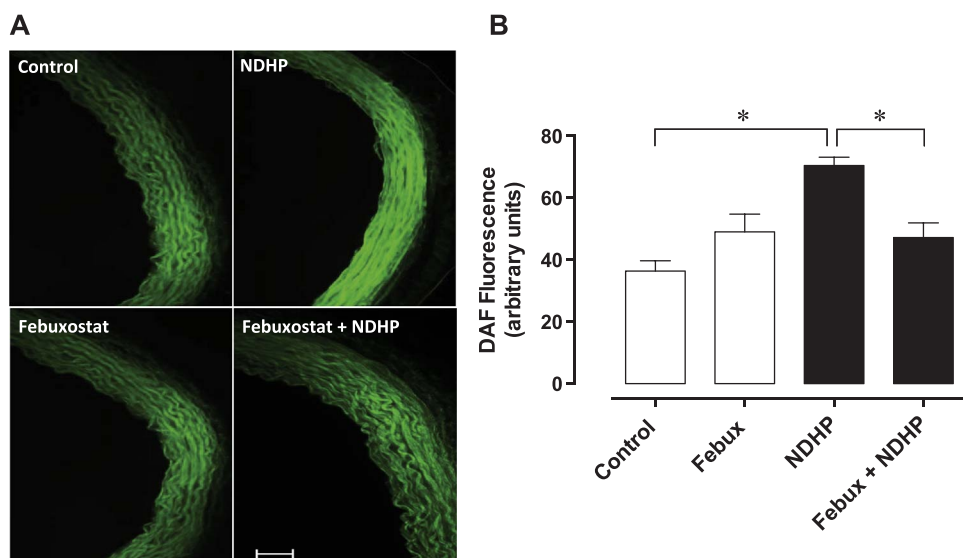
### 3.2. NDHP-mediated nitric oxide fluorescence in mesenteric arteries is mediated by xanthine oxidoreductase

Fluorescence microscopy images (Fig. 2A) show the changes in the fluorescence emitted by DAF-2 DA, an index of NO production, in response to different treatments. Incubation with DAF-2 DA alone was set as baseline and served as control (Fig. 2B). Incubation with NDHP significantly increased fluorescence compared with control ( $95.7 \pm 3.4$  vs.  $10.9 \pm 0.8$  a.u., respectively,  $p < 0.05$ ). The positive control, SIN-1, also increased DAF-2 DA fluorescence, suggesting increased NO production ( $102.5 \pm 2.1$  vs.  $10.9 \pm 0.8$  a.u., respectively,  $p < 0.05$ ). In addition, L-NNA did not prevent the increase in fluorescence promoted by NDHP ( $94.2 \pm 3.6$  vs.  $95.7 \pm 3.4$  a.u., respectively,  $p < 0.05$ ) or SIN 1 ( $99.8 \pm 1.9$  vs.  $102.5 \pm 2.1$  a.u., respectively,  $p < 0.05$ ). Fluorescence microscopy images (Fig. 3A) show the changes in the

fluorescence emitted by DAF-2 DA during inhibition of XOR with febuxostat. Simultaneous incubation with febuxostat tended to increase the fluorescence in mesenteric arteries. Increased fluorescence following treatment with NDHP was blocked by febuxostat (Fig. 3B).

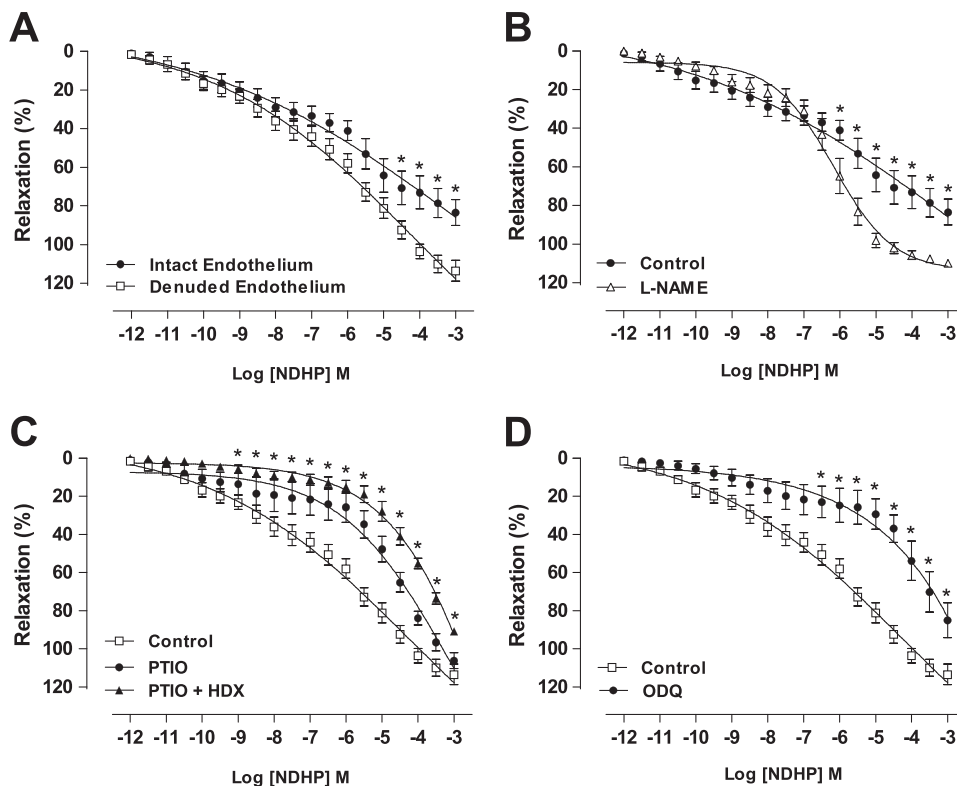
### 3.3. NDHP-mediated vasorelaxation of mesenteric artery – modulation of nitric oxide release and signaling

NDHP ( $10^{-12}$ – $10^{-3}$   $\mu$ M) promoted concentration-dependent vasorelaxation in precontracted mesenteric artery rings isolated from normotensive rats in the presence of functional endothelium ( $83.3 \pm 6.7\%$ ; n = 6). After removal of the endothelium, vasorelaxation was potentiated ( $113.4 \pm 5.3\%$ , n = 7) (Fig. 4A). There was no significant difference between pD2 for rings with or without endothelium. However, pre-incubation with the NOS inhibitor L-NAME, increased NDHP-induced relaxation ( $109.7 \pm 1.5\%$ ; n = 6) compared with control ( $83.3 \pm 6.7\%$ ; n = 6) (Fig. 4B). Thus suggesting that NDHP-induced relaxation is attenuated by eNOS participation. Therefore, in all subsequent experiments the investigation of NDHP-mediated signaling was performed after removal of the vascular endothelium.



**Fig. 3.** NDHP-mediated generation of nitric oxide species in small arteries involves xanthine oxidoreductase. (A) Representative images of superior mesenteric artery sections incubated with the fluorescent cell permeable NO probe DAF-2 DA in the absence or presence of simultaneous treatment with febuxostat and/or NDHP. (B) Bar graph showing the NO production after incubation with only DAF-2 DA (Control), DAF-2 DA + Febuxostat, DAF-2 DA + NDHP and DAF-2 DA + NDHP + Febuxostat. Fluorescence was quantified by optic densitometry (arbitrary units). Data are shown as mean  $\pm$  SEM. n = 13–16 per group. \*p < 0.05 between indicated groups.





**Fig. 4.** NDHP-mediated vasorelaxation and the role of nitric oxide. Concentration-response curves were induced by cumulative addition of NDHP ( $10^{-12}$ – $10^{-3}$  M) in PHE-precontracted (10  $\mu$ M) superior mesenteric artery rings. Panel A shows NDHP inducing concentration-dependent vasorelaxation in both, intact and denuded-endothelium vessel segments ( $*p < 0.05$  versus intact endothelium). Panel B shows the effect of NOS inhibition by L-NAME in intact-endothelium vessel segments ( $*p < 0.05$  comparing to intact-endothelium control segments). Panel C shows the effect of NO scavenger PTIO alone or in combination with HDX in NDHP inducing vasorelaxation ( $*p < 0.05$  for denuded-endothelium pretreated with PTIO + HDX versus denuded-endothelium control). Panel D shows the effect of sGC inhibitor ODQ in the vasorelaxation elicited NDHP ( $*p < 0.05$  versus denuded-endothelium). Data are shown as mean  $\pm$  SEM.  $n = 6$ – $8$  per group.

In precontracted mesenteric artery rings and pre-incubated with the NO scavenger PTIO, the concentration-response curve was shifted to the right, decreasing the NDHP potency when compared to the control ( $pD_2 = 5.1 \pm 0.1$  vs.  $6.5 \pm 0.1$ ,  $n = 6$ ,  $p < 0.05$ ) (Fig. 4C). However, when the rings were preincubated with PTIO together with hydroxocobalamin (HDX), the right shift of the concentration-response curve was increased with reduced maximum effect compared to the control curve ( $90.8 \pm 0.8$  vs.  $113.4 \pm 5.3\%$ ,  $n = 6$ ,  $p < 0.05$ ). Moreover, the sGC inhibitor ODQ decreased NDHP-mediated vasorelaxation compared to the control ( $84.9 \pm 9.5$  vs.  $113.4 \pm 5.3\%$ ,  $n = 6$ ,  $p < 0.05$ ) (Fig. 4D). Taken together, the data suggest that NDHP-induced vasodilation can be mediated by NO release and activation of cGMP signaling.

### 3.4. NDHP-mediated vasorelaxation of mesenteric artery – modulation of $K^+$ channels

Pre-incubation of mesenteric artery rings with TEA, in a concentration that predominantly acts as a non-selective  $K^+$  channels blocker, shifted the concentration-response curve to the right, decreasing the NDHP potency (Fig. 5A), shown by  $pD_2$  value when compared with the control ( $pD_2 = 4.8 \pm 0.1$  vs.  $6.5 \pm 0.1$ ,  $n = 6$ ,  $p < 0.05$ ). The curve displacement by NDHP was also present when  $K^+$  channel subtypes were evaluated using a threefold lower concentration of TEA to selectively block the voltage-gated and  $Ca^{2+}$ -activated potassium channels ( $BK_{Ca}$ ) ( $pD_2 = 5.1 \pm 0.1$  vs.  $6.5 \pm 0.1$ ,  $n = 6$ ,  $p < 0.05$ ) (Fig. 5B), or with inhibition of voltage-gated potassium channels ( $K_v$ ) with 4-AP ( $pD_2 = 5.7 \pm 0.1$  vs.  $6.5 \pm 0.1$ ,  $n = 7$ ,  $p < 0.05$ ). On the other hand, vasorelaxation elicited by NDHP was not altered by inhibition of ATP-sensitive potassium channels ( $K_{ATP}$ ) using GLIB (Fig. 5D) or by inhibition of inward-rectifier potassium channels ( $K_{IR}$ ) with  $BaCl_2$  (Fig. 5E).

### 3.5. Acute preclinical toxicity of NDHP

Oral administration of NDHP (300 mg/kg) did not evoke any

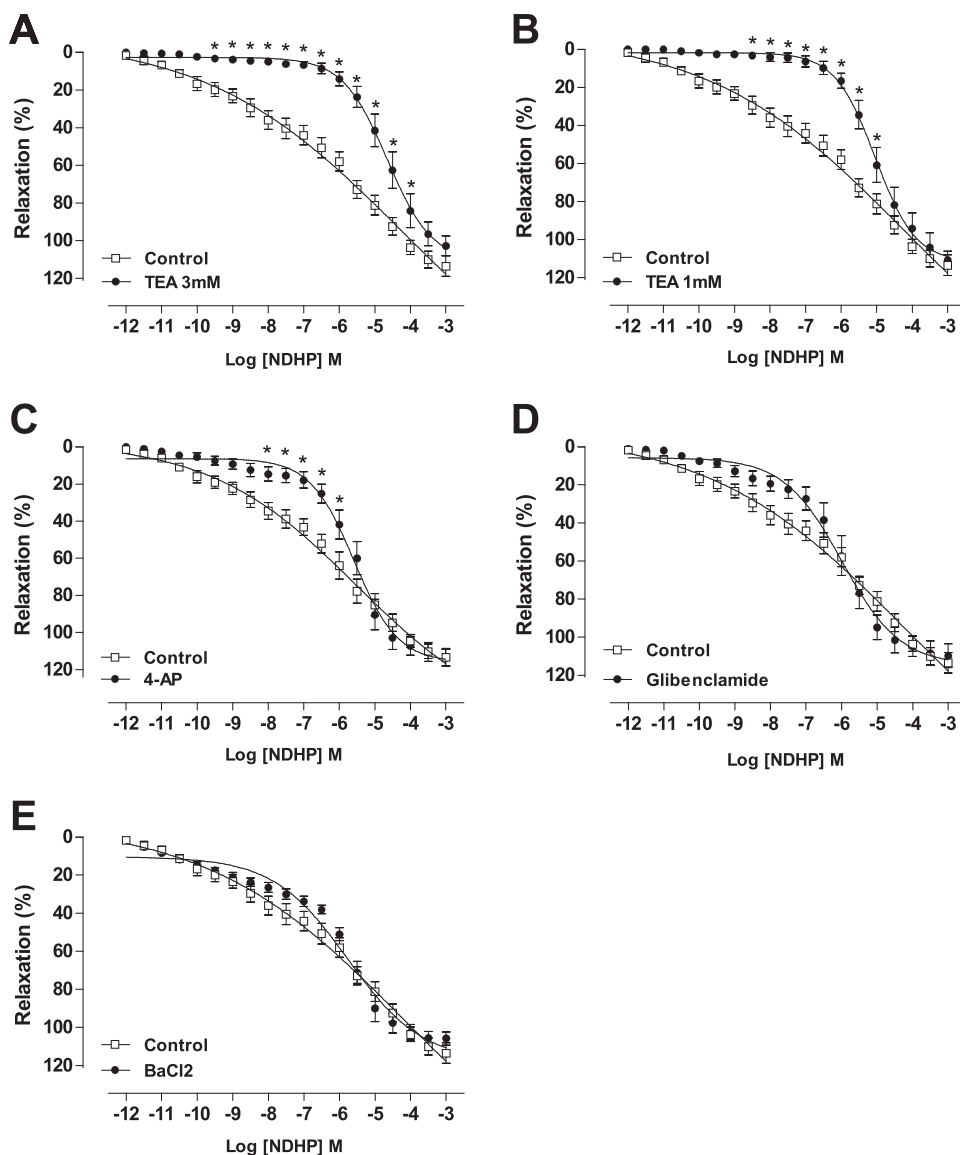
behavioral changes compared to the control group. However, animals treated with the much higher dose (2000 mg/kg) presented analgesia and decreased ambulation, when compared to the control. Regarding the  $LD_{50}$  assessment, no death was observed in animals treated with 300 and 2000 mg/kg of NDHP. Thus,  $LD_{50}$  for NDHP is around 5000 mg/kg (category 5). Treatment with NDHP in these two doses did not promote any changes of body weight and organs weights, as well as, on consumption of water and food compared with the control group (Table 1).

### 3.6. Acute treatment with NDHP reduces blood pressure in 2K1C hypertensive rats

Six weeks after 2K1C surgery, baseline MAP was increased compared with sham-operated rats ( $172 \pm 10$  vs.  $117 \pm 2$  mmHg,  $n = 8$ ,  $p < 0.05$ ) as shown by representative tracings from sham-operated and 2K1C-hypertensive rats (Fig. 6A). In normotensive rats, acute intravenous administration of NDHP (1; 5; 10; 20 mg/kg) decreased blood pressure ( $-11 \pm 1$ ;  $-19 \pm 2$ ;  $-28 \pm 2$ ;  $-44 \pm 5$  mmHg, respectively) (Fig. 6B). NDHP (1; 5; 10; 20 mg/kg) reduced blood pressure dose-dependently in conscious hypertensive rats ( $-16 \pm 3$ ;  $-23 \pm 4$ ;  $-50 \pm 1$ ; and  $-71 \pm 8$  mmHg, respectively) (Fig. 6B).

### 3.7. Chronic treatment with NDHP attenuates the progression of L-NAME and ANG II-induced hypertension

Before any treatment was started (i.e., Baseline), there were no differences in body weight (Supplemental Fig. 1A), blood pressure or heart rate between the vehicle group (MAP:  $98 \pm 2$  mmHg, HR:  $366 \pm 14$  bpm) and the NDHP group (MAP:  $99 \pm 3$  mmHg, HR:  $349 \pm 16$  bpm). Chronic treatment with L-NAME and ANG II significantly increased blood pressure in both groups compared with baseline ( $\Delta MAP_{vehicle}$ : 49 mmHg;  $\Delta MAP_{NDHP}$ : 18 mmHg). However, simultaneous treatment with NDHP significantly attenuated the development of hypertension (Fig. 7A–D). A significant reduction of heart rate was observed in the vehicle group, and a clear trend was observed



**Fig. 5.** NDHP-mediated vasorelaxation and the role of potassium channels. Vasorelaxation induced by cumulative addition of NDHP ( $10^{-12}$ – $10^{-3}$  M) in PHE-precontracted ( $10 \mu\text{M}$ ) superior mesenteric artery rings. (A) Denuded Endothelium vs. Denuded Endothelium + TEA (3 mM). (B) Denuded Endothelium vs. Denuded Endothelium + TEA (1 mM). (C) Denuded Endothelium vs. Denuded Endothelium + 4-AP. (D) Denuded Endothelium vs. Denuded Endothelium + GLIB. (E) Denuded Endothelium vs. Denuded Endothelium +  $\text{BaCl}_2$ . Data are shown as mean  $\pm$  SEM.  $n = 6$ – $8$  per group. \* $p < 0.05$  versus Control.

**Table 1**  
Acute preclinical toxicity test for NDHP in rats.

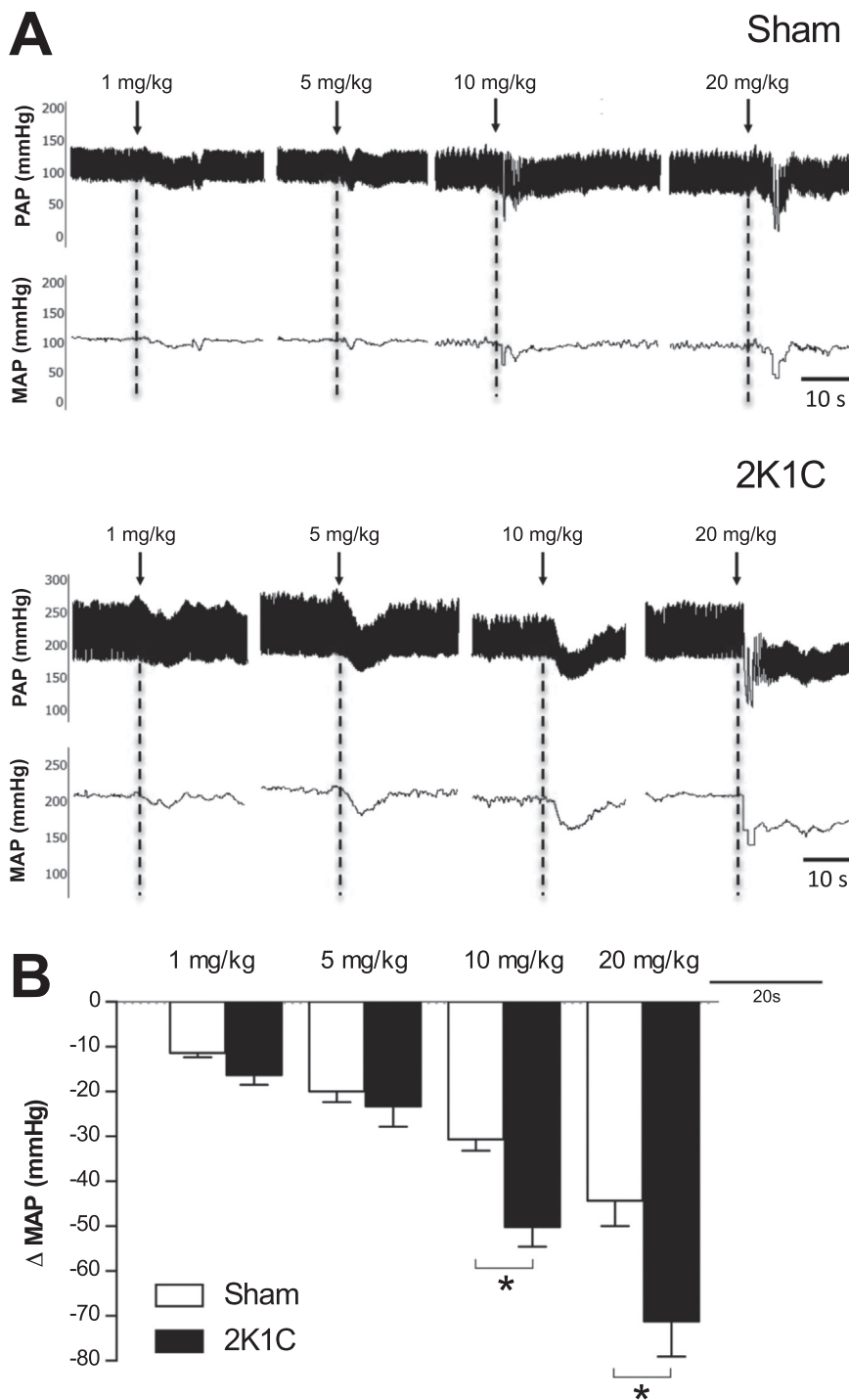
Parameter	Treatment		
	Control	NDHP (300 mg/kg)	NDHP (2000 mg/kg)
<b>Body weight (g)</b>			
Initial	151 $\pm$ 4.50	154 $\pm$ 5.11	162 $\pm$ 3.50
Final	183 $\pm$ 4.48	182 $\pm$ 5.00	185 $\pm$ 6.46
<b>Organ to body weights (mg/g bw)</b>			
Liver	38.6 $\pm$ 1.19	41.7 $\pm$ 1.42	38.4 $\pm$ 1.15
Heart	4.38 $\pm$ 0.18	4.16 $\pm$ 0.09	4.17 $\pm$ 0.19
Kidneys	8.23 $\pm$ 0.29	8.47 $\pm$ 0.22	8.24 $\pm$ 0.18
Spleen	2.71 $\pm$ 0.23	2.62 $\pm$ 0.12	2.59 $\pm$ 0.16
<b>Water intake (mL/rat/day)</b>	29.1 $\pm$ 0.56	30.6 $\pm$ 0.87	29.2 $\pm$ 0.90
<b>Food intake (g/rat/day)</b>	17.0 $\pm$ 0.30	17.6 $\pm$ 0.19	17.1 $\pm$ 0.46

Data are shown as mean  $\pm$  SEM.  $n = 6$  per group. \* $p < 0.05$  compared to Control (vehicle; Cremophor 5%). For organ evaluation, values were expressed as organs index corresponding to division of organ weight (mg) by the body weight of the animal (g).

in the NDHP treated rats (Supplemental Fig. 1B). No differences in heart and kidney weights were observed between the vehicle and NDHP groups following chronic treatment with L-NAME and ANG II. Plasma nitrate was significantly higher in NDHP-treated rats with L-NAME + ANG II compared with placebo-treated rats ( $21.6 \pm 2.9$  vs  $4.4 \pm 1.1 \mu\text{M}$ ,  $p < 0.05$ ), whereas no significant difference was observed for nitrite ( $0.416 \pm 0.156$  vs  $0.179 \pm 0.051 \mu\text{M}$ ) (Supplemental Fig. 2).

### 3.8. Chronic treatment with NDHP attenuates the development of endothelial dysfunction in rats with L-NAME + ANG II-induced hypertension

The contraction of mesenteric resistance arteries in response to PHE and ANG II were similar between NDHP- and vehicle-treated rats (Supplemental Fig. 3A–B). The sensitivity to ACh was significantly reduced in vehicle-treated rats compared with NDHP treatment (Fig. 8A), whereas similar vasorelaxation was observed to the NO donor SNP (Fig. 8B). Taken together, these findings suggest that NDHP, at least in part, can preserve endothelial function in this experimental model of hypertension.



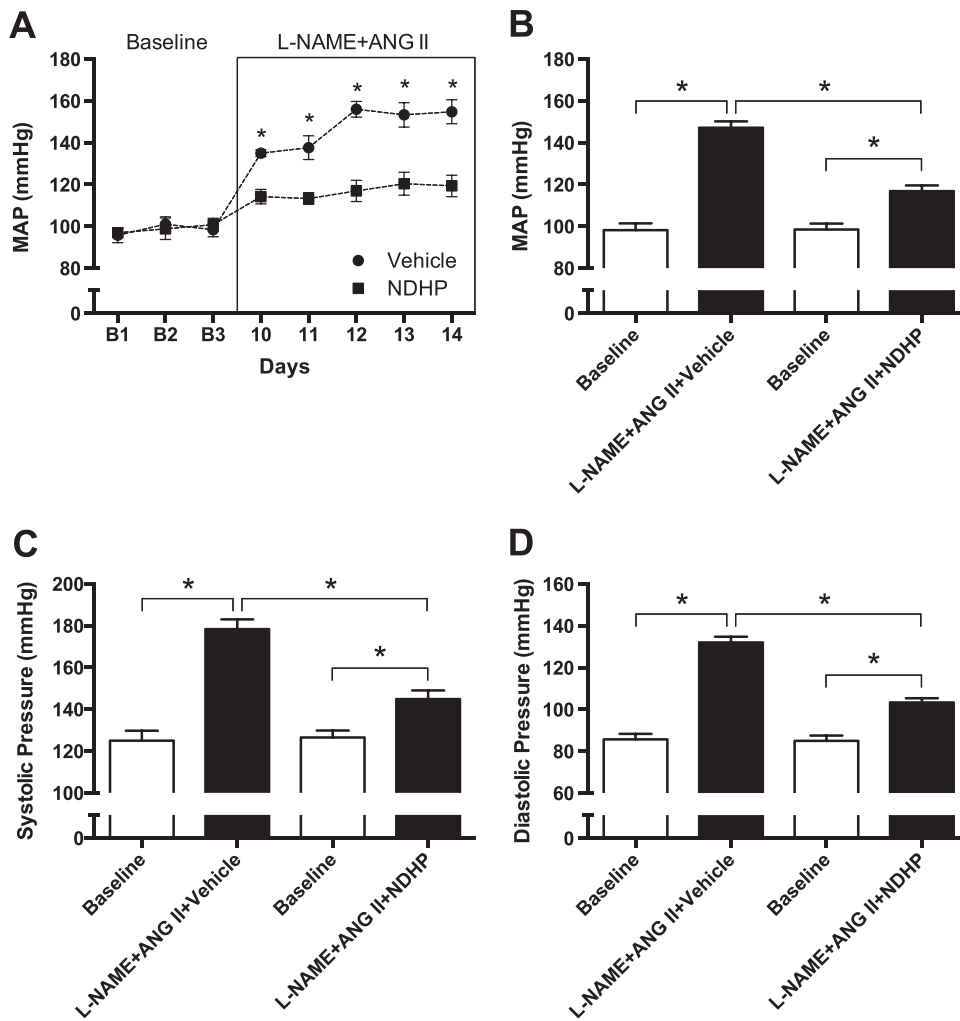
**Fig. 6.** Acute cardiovascular effects of NDHP in normotensive and hypertensive rats. Representative tracings (A) and grouped data on changes in mean arterial pressure ( $\Delta$  MAP) in normotensive sham-operated and hypertensive-2K1C rats (B) following intravenous administration of NDHP (1, 5, 10 and 20 mg/kg). Data are shown as mean  $\pm$  SEM.  $n = 8$  per group. \* $p < 0.05$  between indicated groups.

#### 4. Discussion

Cardiovascular disease, including hypertension, is the main cause of death worldwide, and the therapeutic value of new strategies to increase NO signaling in the cardiovascular system have previously been discussed [35]. Several NO donors and organic nitrates have been studied in the past [16–22], but have been associated with adverse effects mainly due to short half-life and unspecific location for NO release, as well as the development of tolerance with repeated dosing [35,36]. Due to the need for new NO donors with better pharmacological properties, we recently synthesized and characterized 1,3-bis(hexyloxy)propan-2-yl nitrate (NDHP) [27]. This novel organic mononitrate generates NO, which is catalyzed by XOR, and the effects of

NDHP were not subjected to development of tolerance [27]. The present study extends our previous findings, demonstrating that acute treatment with NDHP mediated vasorelaxation and significant reduction of blood pressure in hypertensive animals. Moreover, we show that chronic treatment with NDHP can attenuate the progression of hypertension and endothelial dysfunction in a model of cardiovascular disease.

In this study we further evaluated the underlying mechanism(s) of NO formation and investigated the acute and chronic effects of NDHP in two animal models of hypertension (*i.e.* 2K1C and L-NAME + ANG II). Optical measurement techniques of NO using diaminofluorescein (DAF) or other NO-sensitive dyes are useful in locating anatomically where NO is being produced [37]. Based on the assumption that the

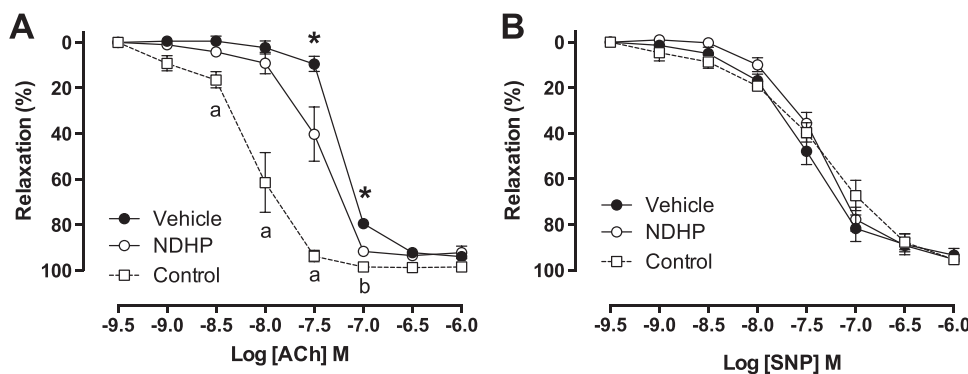


**Fig. 7.** Cardiovascular effects of NDHP in a model of hypertension in rats. Effects of chronic treatment with NDHP in an *in vivo* model of L-NAME and ANG II-induced hypertension. Mean arterial pressure (A-B), systolic pressure (C) and diastolic pressure (D) during baseline (B1-B3) and during treatment with NOS inhibition (L-NAME, 0.5 g/L in drinking water) and infusion with angiotensin II (ANG II; 120 ng/kg/min) on Days 10–14. Data are shown as mean  $\pm$  SEM (n = 8/group). In Panel A, \*p < 0.05 between vehicle and NDHP group. In Panels C-D, \*p < 0.05 between the indicated groups.

NDHP may generate NO *in vitro*, we quantified the intracellular NO production in vascular smooth muscle cells (VSMCs) of small mesenteric artery from normotensive rats using the probe DAF-2 DA. Mesenteric artery cross sections, stimulated with NDHP and incubated with DAF-2 DA, showed increased fluorescence emitted by the probe. Since the vascular endothelium is the main physiological source of NO, the eNOS blocker L-NNA was used to investigate the possible participation of this enzyme in NDHP-mediated NO production. Pre-treatment with L-NNA did not prevent the increase of fluorescence emission in both NDHP-treated tissues and tissues treated with another known NO donor, SIN-1.

Next we showed that NDHP promoted vasodilatation of small mesenteric arteries from normotensive rats. Our data showed that NDHP-

mediated vasorelaxation was potentiated in vessels without endothelium or when eNOS was blocked with L-NAME. This interaction requires further investigation, but suggests that eNOS negatively modulates the effect of NDHP. This is in agreement with a previous study by Potje et al. [38], showing that endothelium removal or pharmacological inhibition of NOS increases the effects of the NO donor TERPY (metal complexes of ruthenium) in Wistar rat aortas, but not in spontaneous hypertensive rats (SHR). In addition, we speculate that by removing the endothelium, NADPH oxidase(s) expressed on endothelial cells are also removed, therefore basal superoxide generation is reduced and the bioavailability of NO donated by the compound is increased. In fact, it might not be only the presence of eNOS per se, but rather the lack of NADPH oxidase that influence the profile of the vasorelaxation



**Fig. 8.** Vascular responses in rats with L-NAME and ANG II-induced hypertension. Concentration-response curves in small mesenteric artery rings from healthy rats (Control) and rats treated with L-NAME and ANG II together with either vehicle or NDHP for two weeks. Acetylcholine-mediated vasorelaxation was stronger in rings from NDHP treated rats compared with vehicle (A), whereas no differences were observed in response to sodium nitroprusside (SNP) (D). Results are expressed as % of phenylephrine-mediated contraction, and shown as mean  $\pm$  SEM. n = 7–8 per group. \*p < 0.05 between NDHP versus vehicle. a p < 0.05 between Control and both vehicle or NDHP groups; b p < 0.05 between Control and vehicle group.



curves elicited by NDHP. Taken together, our results show that NDHP-mediated NO release and vasorelaxation is endothelium-independent, which may be associated with favorable effects during cardiovascular disease when eNOS function is compromised.

Our previous and current data indicate that NDHP undergoes a metabolism process with consequent release of NO in VSMCs without the participation of eNOS. However, it is not possible from our *ex vivo* vessel studies with the DAF-FM probe to determine the absolute NO levels due to several technical limitations, such as irregular distribution of DAF in tissues or cells, interference from other chemical compounds, photoactivation and photobleaching [39–41]. Some NO-like compounds release NO spontaneously or non-enzymatically, while others require enzymatic catalysis to release NO [42]. Several enzymes play important biological roles in the release of NO from NO-donors, including, aldehyde oxidase and XOR [43,44]. In agreement with our previous study [27] the current study demonstrates that NDHP-mediated NO production required functional XOR activity.

Next we aimed to further investigate the underlying mechanism for endothelium-independent vasorelaxation by NDHP. VSMCs express several plasma membrane  $K^+$  channels, which may be modulated by NO. Organic nitrates mediate NO release in VSMCs and can activate the sGC-cGMP-PKG pathway leading to hyperpolarization and consequently decreased  $Ca^{2+}$  in the cell [45,46]. Important targets of PKG which promote relaxation of VSMCs are the  $K^+$  channels, including voltage-dependent  $K^+$  channels ( $K_v$ ), large-conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ), inwardly-rectifying  $K^+$  channels ( $K_{IR}$ ), ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels [47–50]. In order to investigate the involvement of  $K^+$  channels, preparations of mesenteric artery were incubated with above mentioned  $K^+$  channels inhibitors. Using TEA (3 mM), a decrease in the potency of NDHP was observed, suggesting that some of these channels may be involved in the compound-induced vasorelaxant effect. From these results we set out to investigate the different subtypes of  $K^+$  channels by using specific blockers. As manifested by the rightward shift of the concentration-response curve, TEA (1 mM) and 4-AP (1 mM) decreased the effect of NDHP. This suggested participation of  $K_v$  and  $BK_{Ca}$  channels, respectively. Conversely, GLIB or  $BaCl_2$  did not influence the vasorelaxant effect induced by NDHP, demonstrating no involvement of the  $K_{ATP}$  or the  $K_{IR}$  channels.

These promising *ex vivo* findings with NDHP encouraged *in vivo* studies to assess the potential therapeutic effects in models of hypertension. Increasing doses of NDHP were used to evaluate the acute cardiovascular effects in normotensive animals and in rats with 2K1C-induced hypertension. Renovascular hypertension is considered to be the most frequent cause of secondary hypertension. The 2K1C renovascular hypertension model is associated with increased plasma renin activity and circulating ANG II concentration, increased sympathetic tone, uncoupling of eNOS and oxidative stress in specific organs [51–53]. Our findings showed that intravenous administration of NDHP reduced blood pressure in a dose-dependent manner in both groups. However, the observed blood pressure-lowering effect was more pronounced in hypertensive animals.

In order to assure safety for NDHP treatment an acute toxicity test was performed. Two high doses of NDHP were selected in this toxicity study (*i.e.* 300 and 2000 mg/kg body weight), to investigate the risk of lethality or the presence of any adverse effects. According to the behavioral parameters evaluated, it was observed that NDHP presented analgesia and decreased ambulation at the highest dose. Studies have shown that NO has important actions in the central nervous system. For example NO influences peripheral somatosensory actions antinociceptive by acting on specific channels in neurons [54,55], which may explain the NDHP-mediated CNS depressant effect at higher doses. No death of the animals was observed for any of the tested doses, and NDHP had no significant effect on the body weight development, food or water intake, or on organ weights during the 14 days of evaluation compared with the placebo-treated control group.

Since our toxicity studies did not reveal any adverse effects of

NDHP, we finally assessed the therapeutic value of chronic treatment with NDHP in an experimental model of endothelial dysfunction and hypertension. Supporting the acute blood pressure lowering effects of NDHP, we also observed that chronic treatment with NDHP attenuated L-NAME and ANG II induced hypertension. As expected, markers of NO homeostasis (*i.e.* nitrate and nitrite) were increased in rats treated with NDHP, which is consistent with the idea of NO generation from NDHP. Moreover, our findings from isolated vessels suggested that chronic treatment with NDHP, at least in part, prevented the development of endothelial dysfunction in this disease model.

Limitations of the current study include the use of different administration routes for the acute and chronic studies, the use of a single species (rat) and that additional doses of NDHP will be necessary to investigate its favorable cardiovascular effects. Future investigations are warranted to investigate the role of XOR in mediating the bioactivation of NDHP *in vivo*, and also to elucidate the role of different  $K^+$  channels modulating the vasorelaxant and antihypertensive effect of NDHP.

In conclusion, our data show that NDHP is a novel NO donor that induces vasorelaxation via activation of NO/cGMP/PKG pathway and specific  $K^+$  channels ( $K_v$  and  $BK_{Ca}$ ), and also exerts blood pressure lowering effects in hypertensive rats. The therapeutic value of this new organic mononitrate will be further evaluated in additional experimental studies and in planned clinical trials.

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## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2017.12.004>.

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