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Tetrahydrofurfuryl Nitrate: A New Organic Nitrate Induces Hypotension and Vasorelaxation Without Vascular Tolerance Induction

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Abstract: The development of new organic nitrates is still relevant due to the clinical limitations of their use. Tetrahydrofurfuryl nitrate (NTHF) is a new organic nitrate obtained through a synthetic route of sugarcane. The aim of this research was to investigate the cardiovascular effects promoted by NTHF in rats. Isolated vascular smooth muscle cells (VSMC) were incubated with a specific probe and were analyzed in a flow cytometer to measure the NO concentration after NTHF treatment. Rat superior mesenteric rings were isolated and used for isometric tension recordings and the evaluation of the vasorelaxant activity induced by NTHF. For the in vivo study, polyethylene catheters were implanted into the abdominal aorta and inferior vena cava of the rats (weighing 250–300 g). NTHF increased NO levels in rat VSMCs. In anesthetized rats, NTHF induced hypotension and bradycardia after intravenous administration. These effects were attenuated after the administration of a sGC inhibitor, methylene blue. In the phenylephrine pre-contracted superior mesenteric artery of rats, NTHF (1 pM–10 μM) induced concentration-dependent vasodilatation in both the intact and removed endothelium. Furthermore, in the presence of NO° scavenging (C-PTIO and HDX) or ODQ, a sGC inhibitor, the vasorelaxation induced by NTHF was decreased. NTHF tolerance was evaluated in mesenteric artery rings previously exposed with isolated concentrations of the new organic nitrate. The vasorelaxant effect was not modified by exposure to nitrate. These results demonstrated that NTHF induced hypotension and bradycardia in vivo and a vasorelaxant effect with the participation of the NO-sGC-PKG pathway and triggering calcium-activated K^+ channels without vascular tolerance induction.

Keywords: blood pressure; hypotension; nitric oxide donor; organic nitrate; potassium channels; vasodilation

1. Introduction

Nitric oxide (NO) is a free radical and a gaseous molecule that plays a crucial role in the maintenance of the cardiovascular system, such as vascular tone, vascular remodeling and the inhibition of platelet aggregation [1,2]. The NO effects are mediated by the activation of the soluble guanylyl cyclase (sGC) enzyme, which increases 3′,5′-cyclic guanosine monophosphate (cGMP) production [3,4].

NO is highly reactive, possesses a short half-life and it is responsible for mediating various processes in the cardiovascular system such as the endothelium-dependent vasorelaxation, platelet adhesion and aggregation and the regulation of baseline blood pressure (BP) [5–7].

The biosynthesis of NO occurs by the oxidation of L-arginine catalyzed by nitric oxide synthase (NOS), which can be found in three isoforms: the inducible form (iNOS), neuronal (nNOS) and endothelial NOS (eNOS). NOS isoforms use L-arginine as the substrate, require molecular oxygen for the reaction and the co-factors include flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and 6R-5,6,7,8-tetrahydrobiopterin (BH_4) [8]. However, when BH_4 is deficient due to oxidative inactivation, the dimer of NOS breaks down, generating ROS (especially $O2^{\bullet -}$) instead of NO [9–12]. This state is referred to as eNOS uncoupling and also happens downstream of NADPH activation [10,12]. In addition, the increase in ROS production can react with NO and induces peroxynitrite ($ONOO^-$) formation, also diminishing NO bioavailability and cell damage [13,14].

Abnormalities in NO production and/or bioavailability are involved in many diseases, such as hypertension, diabetes and atherosclerosis, as well as in aging [15]. The chemical versatility of NO allows the synthesis of various NO donors, each one with different rates and ways to release the NO molecule [16,17]. NO donors can be divided into direct donors, nitrosothiols and organic nitrates [18].

Organic nitrates, such as nitroglycerin (glyceryl trinitrate, GTN), isosorbide dinitrate (ISDN) and pentaerythritol tetranitrate (PETN), have been used as vasodilators for over a hundred years to improve symptoms in patients with congestive heart failure, stable coronary artery disease, acute coronary syndromes or arterial hypertension [19–21].

GTN and other organic nitrates are prodrugs that require bioconversion to release NO to later induce the production of cGMP through sGC activation [22]. Several enzymes have been related to this bioactivation, such as mitochondrial aldehyde dehydrogenase (ALDH2) [23]. Furthermore, organic nitrates can promote the direct oxidation of G-type protein kinase 1 (PKG1), which induces the activation of this kinase independently of the increase in the cGMP concentration mediated by NO [22,24].

A limitation of some organic nitrates used in the clinic is the induction of tolerance and endothelial dysfunction stimulation under chronic therapy, which result in a rapid reduction in hemodynamic effects [25,26]. Two mechanisms have been proposed to explain the tolerance induced by organic nitrates: tolerance induced by neurohormonal mechanisms and/or by vascular mechanisms. [27]. The vascular mechanisms involve (a) the inhibition of nitrate biotransformation [28], (b) the desensitization of sGC to NO [29], (c) an increase in phosphodiesterase (PDE) activity and (d) the uncoupling of NOS, leading to cross-tolerance to other nitrate-donor substances [30].

Therefore, it is extremely important to search for new organic nitrates with hemodynamic effects, but with a lower tendency to induce tolerance, which led our research group to seek for new compounds such as the organic nitrates [31,32]. It is evident that there is great importance regarding developing new molecules that can overcome the effects of conventional therapy using organic nitrates. Based on this perspective, a new organic nitrate was developed: tetrahydrofurfuryl nitrate (NTHF) (Figure 1).

Among the nitrates researched in Brazil, organic nitrate derived from tetrahydrofurfuryl alcohol was obtained on a large scale, using sugarcane waste as the raw material. This agricultural residue constitutes an economic and sustainable alternative, as it is generated in tons by sugar and alcohol plants. The reaction from biomass consists of a known synthesis route, through the digestion of sugarcane waste, followed by the dehydration of pentoses

to obtain furfural. In the second stage, furfural is converted into tetrahydrofurfuryl alcohol (ATHF) and in the third and the last stage, ATHF is esterified to obtain NTHF, with a yield of 81%. The present study purposed to elucidate the mechanism of action involved in cardiovascular effects induced by NTHF in rats.

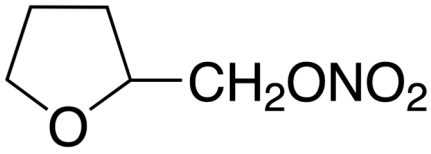

Figure 1. Chemical structure of tetrahydrofurfuryl nitrate (NTHF).

2. Materials and Methods

2.1. Synthesis of NTHF

NTHF was synthesized at the Department of Chemistry at Federal University of Paraíba. It was obtained by a synthetic route through sugarcane bagasse digestion followed by dehydration of pentoses to obtain furfural. Secondly, furfural was converted to tetrahydrofurfuryl alcohol and, lastly, it was esterified to obtain NTHF (Figure 1).

2.2. Drugs and Solutions

The following drugs were used: cremophor EL, acetylcholine hydrochloride (ACh), L phenylephrine chloride (Phe), sodium nitroprusside (SNP), methylene blue (MB), N-acetylcysteine (NAC), 1H-[1–3]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), hydroxocobalamin (HDX), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), tetraethylammonium (TEA), charybdotoxin (ChTX), 4-aminopyridine (4-AP), glibenclamide (GLIB), 4,5-diaminofluorescein diacetate (DAF-2DA), penicillin–streptomycin solution (10,000 units/mL and 10 mg/mL), 7 aminoactinomycin D (7-AAD) and trypsin–EDTA solution (0.5% and 0.02%), which were obtained from Sigma–Aldrich (São Paulo, SP, Brazil). Additionally, Dulbecco's modified eagle's medium (DMEM) (HIMEDIA®, Kelton, PA, USA), fetal bovine serum (FBS) (INVITROGEN®, Waltham, MA, USA), buffer solution preparation (PBS) (INVITROGEN®, Waltham, Massachusetts, USA), heparin sodium salt (Roche® Brazil, São Paulo, Brazil), sodium thiopental and glyceryl trinitrate (GTN) (Cristália, São Paulo, SP, Brazil) were used. NTHF was solubilized in a mixture of distilled water and cremophor at a concentration of 10 mM and diluted to the desired concentration with distilled water just before use. ODQ and glibenclamide were dissolved in DMSO, while C-PTIO was dissolved in absolute ethanol and diluted in distilled water and all other compounds were dissolved only in distilled water. The final concentration of cremophor and DMSO in the organ bath never exceeded 0.01%. The solutions were stored at −4 to 0 °C. The composition of Tyrode's solution was (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. The composition of the HANK solution was (mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.3; $MgCl_2$, 1.0; NaH_2PO_4, 0.3; $NaHCO_3$, 4.2; glucose, 5.0; $MgSO_4$, 0.8; and KH_2PO_4, 0.4. The composition of Hank's solution was (mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.3; $MgCl_2$, 1.0; NaH_2PO_4, 0.3; $NaHCO_3$, 4.2; glucose, 5.0; $MgSO_4$, 0.8; and KH_2PO_4, 0.4.

2.3. Animals

In all experiments, male Wistar rats (200–300 g) housed under conditions of controlled temperature (21 ± 1 °C) and lighting (light on 6 a.m.–6 p.m.) with access to food and tap water ad libitum were used. The experimental approaches were carried out after approval by the Animal Care and Use Committee of the Federal University of Paraíba (CEUA/UFPB—protocol # 0310/09 and 0207/12).

2.4. Culture of Vascular Smooth Muscle Cells (VSMC) from Rat Aorta

The isolation of VSMC was carried out from the explant technique. A segment of approximately 3 cm from the thoracic aorta was collected and transferred to a 15 mL sterile tube containing Hank's solution and antibiotics. The fragments were placed with the luminal part face down on the plate wells for cell culture. On each fragment was placed 50 µL of DMEM supplemented with 10% FBS and antibiotics. The preparations were maintained in a tissue culture incubator at 5% and 37 °C and humidity of 90% by exchanging the culture medium every day. The VSMC migration occurred between the seventh and tenth day of culture. After 30% of board occupation, the explants were removed. When 90% of confluence was reached, the cells were dispersed by treatment with trypsin solution (0.5%)/EDTA (0.02%) for 3 min in an oven of CO_2. The obtained suspension was collected and centrifuged for 5 min at 2000 rpm. The supernatant was discarded and added to DMEM supplemented with FCS (20%) plus antibiotics to the pellet. Then, the cells were distributed into new cell culture plates [31].

The myocytes' confluence (90%) was released by trypsinization and suspended in DMEM plus 20% FBS, which was centrifuged at 2000 rpm for 5 min. Then, the supernatant was discarded and the pellet was resuspended in Hank's solution. An aliquot was designed to test cell viability with 7-aminoactinomycin D (7-AAD) by flow cytometry.

2.5. NO Measurement in Aortic VSMC

The analyses were performed with a FACS Canto II from Becton-Dickinson (San Jose, CA, USA) equipped with an argon laser emitting a beam of 488 nm. Initially, 10^6 cells per ml were analyzed in the flow cytometer in the absence of fluorescent probes and drugs (unlabeled cells) in order to observe the parameters of cytometry and cell autofluorescence. Then, cells were incubated with the specific probe NO DAF-2DA (4,5-diaminofluorescein diacetate 10 mM) for 30 min and, immediately afterward, analyzed. Then, the vehicle or GTN (10 µM) or SNP (10 µM) or NTHF (100 µM) were added separately to DAF-loaded cells (106 cells/mL). After treatment, the samples were analyzed separately for 30 min at 10 min intervals. The acquisition for each sample was 10,000-cells hydrodynamic flow and the average intensity of the emitted fluorescence (FITC obtained channel whose wavelength range is 515–545) was measured using DIVA software version 4.0 (Becton-Dickson San Jose, CA, USA). Data were expressed as the change (delta) fluorescence percentage, normalized by the fluorescence emitted in the presence of DAF vehicle. To this end, the fluorescence obtained in the presence of DAF plus vehicle was termed "control" and that obtained in the presence of DAF more DRUG was called "treated" according to the following equation: (Δ%) fluorescence = (TREATED–CONTROL) × 100/CONTROL.

2.6. Surgical Procedures and In Vivo Protocol

For the measurement of arterial blood pressure, a technique described by Queiroz, et al. was used [33]. Briefly, under sodium thiopental anesthesia (45 mg/kg, i.v.), the lower abdominal aorta and inferior vena cava were cannulated via the left femoral artery and vein using polyethylene catheters. Then, catheters were filled with heparinized saline solution and tunneled under the skin to emerge between the scapulae. The BP was measured 24 h after surgery by connecting the arterial catheter to a pre-calibrated pressure transducer (MLT0380/D, ADInstruments, Sidney, Australia) connected to a data acquisition system (Mikro-tip Blood pressure system, ADInstruments, Sidney, Australia) running the LabChart software (version Pro 7.0, ADInstruments, Sidney, Australia). The data were sampled at 2000 Hz. For each pulse pressure, the computer calculated mean arterial pressure (MAP) and heart rate (HR). In a group of conscious rats, MAP and HR were recorded before (baseline) and after intravenous bolus administration of NTHF (10, 20, 30, 40 and 50 mg/kg body weight, i.v., randomly). Afterwards, i.v. injection of methylene blue (3 mg/kg), a sGC blocker, was administered to the animals 5 min before the random administration of NTHF.

2.7. Tissue Preparation and In Vitro Protocol

Rats were euthanized by stunning and exsanguination. The superior mesenteric artery was removed, placed in Tyrode's solution and dissected in order to make it free of adhering tissue. It was sectioned in rings (1–2 mm), which were suspended by cotton threads in organ baths containing 10 mL of Tyrode's solution and maintained at 37 °C for isometric tension recordings. The stabilization period was 1 h under a resting tension of 0.75 g. During this time, the solution was changed each 15 min to prevent the accumulation of metabolites. The isometric tension was recorded by a force transducer (MLT020, ADInstruments, Sidney, Australia) coupled to an amplifier recorder (ML870/P with LabChart version 7.0, ADInstruments, Sidney, Australia). The endothelium was mechanically removed by gently rubbing the vessel intima with a wire. The presence of functional endothelium was assessed by the ability of acetylcholine (ACh) (10 μM) to induce more than 90% relaxation of precontracted vessels with Phe (10 μM). The absence of the relaxation to ACh was taken as evidence that the vessel segments were functionally denuded of endothelium.

2.8. Investigation of the Vasorelaxant Effect of NTHF

To study the vasodilator effects of NTHF, isolated mesenteric artery rings were previously contracted with Phe (1 μM). Under this condition, the vessels were exposed to cumulative concentrations of NTHF; increasing concentrations were added (10^{-12}–10^{-5} M) in a cumulative manner to obtain a concentration–response curve in the presence or absence of endothelium. The response was expressed as a percentage of relaxation with respect to contraction produced by Phe. The vasodilation induced by the nitrate was examined in the presence of different pharmacological tools. To examine whether NO/sGC pathway was the target of NTHF vascular action, mesenteric rings without endothelium were preincubated for 30 min with inhibitors after being preconstricted with Phe (1 μM). PTIO (300 μM) is a free radical form of NO (NO•) scavenger, hydroxocobalamin is a NO• scavenger (HDX 30 μM) and ODQ (10 μM) is a soluble guanylyl cyclase (sGC) inhibitor. In addition, to assess the involvement of K+ channels, different blockers were also studied, a non-selective K+ channels blocker with TEA (3 mM), TEA (1 mM), which in this concentration selectively blocks the BKCa channels, 4-AP (1 mM), GLIB (10 μM) and BaCl2 (30 μM) that block KATP, Kv and KIR channels, respectively, and ChTX (100 nM), a well-known BKCa channels blocker. Furthermore, a ROS scavenger, NAC (3 mM), was also added to the organ baths. All these inhibitors were added 30 min before the application of Phe (1 μM). In the tonic phase of the contraction, NTHF (10^{-12}–10^{-5} M) was cumulatively added to preparations. The inhibition was calculated by comparing the response elicited by NTHF in the absence and presence of inhibitors in the preparation.

2.9. Tolerance Protocol

In order to investigate whether NTHF develops vascular tolerance, the vasorelaxant response was evaluated after repeated exposures of the vessels to this nitrate. Therefore, after verifying the absence of the endothelium, the vessels were incubated with organic nitrate (3, 10, 30 and 100 μM) for 60 min in different experiments in order to mimic a vascular tolerance. After exposure to the tolerance-inducing condition, the vessels were washed repeatedly with Tyrodes's solution and then exposed to cumulative concentration of organic nitrate again and post-incubation-response curves were obtained. Upon reaching 60 min of stabilization, a new contraction was induced with Phe (1 mM), followed by the addition of increasing concentrations of NTHF (1 pM–10 μM) cumulatively to obtain a concentration–response curve. Responses were expressed as a percentage of relaxation according to the contraction produced by the Phe. The potency and efficacy of NTHF vasorelaxation were evaluated using pD2 and MR values, respectively. These values were compared to those obtained in the absence of pre-incubation with NTHF.

2.10. Statistical

All values are expressed as mean ± SEM and individual concentration–response curves were fitted with the Hill logistic equation. The maximum response (MR) was considered as the maximum response of pre-contracted tissues to the highest concentration of NTHF. EC_{50} values were obtained as the concentration at which a half-maximal reduction in tone occurred and the pD2 was calculated as ($-\log EC_{50}$). In each experiment, 'n' indicates the number of rings from different rats. Differences between mean pD2 and MR values were both assessed by unpaired Student's t-test to compare NTHF effects in the absence (control) and presence of the inhibitors. One-way ANOVA followed by Bonferroni's post-test was used in in vivo experiments and for nitric oxide measurement. Data were computed for statistical analysis by using Graph Pad Prism 7.0® version (GraphPad Software, La Jolla, CA, USA) and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Intracellular NO Was Detected in VSMC of Rats After NTHF Incubation

To investigate a possible effect of NTHF on the release of NO, we first measured the NO levels by relative fluorescence after incubation with DAF-2DA. In the presence of 7-AAD, VSMC from rat aorta showed cell viability greater than 90% after incubation with NTHF (10^{-4} M). In rat aorta VSMC, NTHF (10^{-4} M) induced an increase in fluorescence after its addition with DAF-2DA (%fluorescence = 115.5 ± 20; n = 4, $p < 0.05$) and a similar effect was found in the presence of SNP (% fluorescence = 70.56 ± 12; n = 4, $p < 0.05$) and GTN (% fluorescence = 67.07 ± 20; n = 4, $p < 0.05$). The results obtained were compared to a control condition (DAF 10 μM + vehicle) (Figure 2). Additionally, there was no difference between NTHF, SNP and GTN fluorescence.

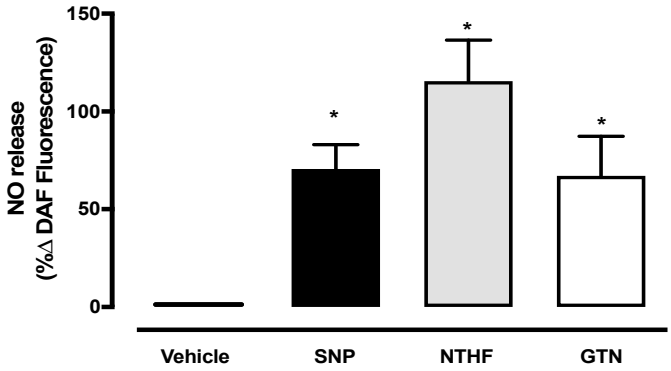

Figure 2. Release of NO by nitrates in rat aortic VSMC. Effect of NTHF (100 μM), SNP (10 μM) and GTN (10 μM) in the generation of NO in rat aortic VSMC, DAF-2DA loaded and analyzed by flow cytometry after 30 min incubation. The percentage of the difference in fluorescence intensity (%), which reflects the increase in the [NO]C, was obtained for each protocol. The data are representative of four experiments (different primary cultures). Values expressed as mean ± E.P.M. * $p < 0.05$ versus vehicle.

3.2. In Vivo Experiments

In order to investigate the response induced by the organic nitrate on cardiovascular parameters, BP and HR were evaluated after the in bolus administration of NTHF (10, 20, 30, 40, 50 mg/kg; i.v.). NTHF elicited dose-dependent hypotensive (6.4 ± 2; 12.28 ± 3.9; 31.15 ± 5.7; 44.10 ± 6; 52.4 ± 4%) and bradycardic effects (4.5 ± 1.7; 11.6 ± 6.8; 41.6 ± 11.3; 79.5 ± 4.4; 88 ± 2%), respectively (Figure 3). The vehicle was also administered and did not promote effects on BP (1 ± 0.6%) and HR (4 ± 1.6%).

The administration of the sGC blocker attenuated the hypotensive (8 ± 4.3; 3.9 ± 1.1; 9.5 ± 2.1; 11.3 ± 5.6; 18.9 ± 10.4%) and bradycardic (4.4 ± 1.4; 5 ± 1.6; 8 ± 2; 22.2 ± 10; 36.4 ± 12%) responses induced by injections of NTHF, respectively, as shown in Figure 3. It suggests the participation of the NO-sGC-PKG pathway.

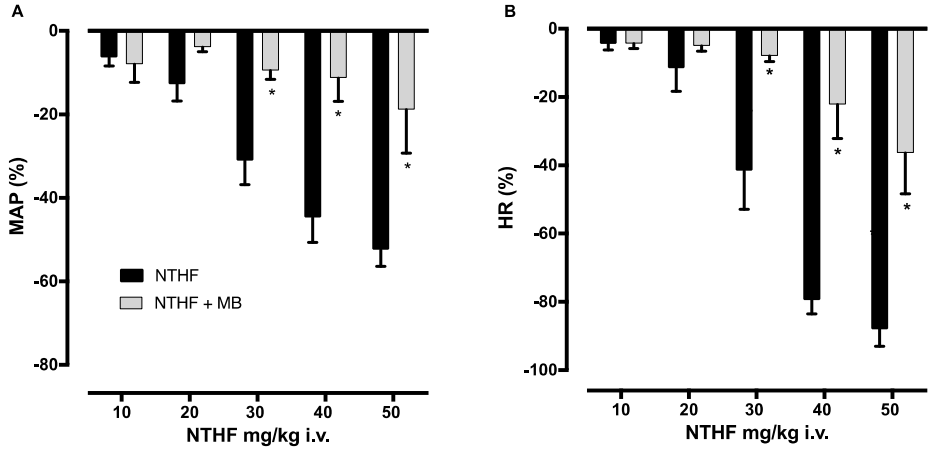

Figure 3. Changes in the arterial mean pressure (MAP) and heart rate (HR) induced by NTHF. Effect promoted by bolus administration of NTHF (10, 20, 30, 40 and 50 mg/kg, i.v.) (n = 6) on MAP (**A**) and HR (**B**) in unanesthetized normotensive rats in the absence or presence of Methylene Blue (3 mg/Kg; i.v.). These data were examined using one-way ANOVA followed by the Bonferroni's post-test. * $p < 0.05$ versus NTHF alone.

3.3. NTHF Induces Endothelium-Independent Vasorelaxation

To determine whether the NO released by NTHF was able to induce vasodilation in the mesenteric artery of rats, we first added ACh to the contraction induced by Phe to verify the presence or absence of the endothelium. Then, the organic nitrate was added to the second pre-contraction produced by Phe. NTHF induced a concentration-dependent vasorelaxation in the intact-endothelium mesenteric artery precontracted with Phe (MR = 84 ± 5.3%, pD2 = 7.86 ± 0.2, n = 10). The mechanical removal of the endothelium did not change the NTHF potency (pD2 = 7.39 ± 0.15; n = 12), but it significantly increased the maximum response (MR = 100 ± 6.1%, $p < 0.05$) (Figure 4). This result suggests that the vasodilator response of the organic nitrate is independent of the relaxing factors derived from the vascular endothelium. Thus, in all subsequent experiments, we used the mesenteric rings without the endothelium to investigate the mechanism of action involved in the vasorelaxant effect evoked by NTHF.

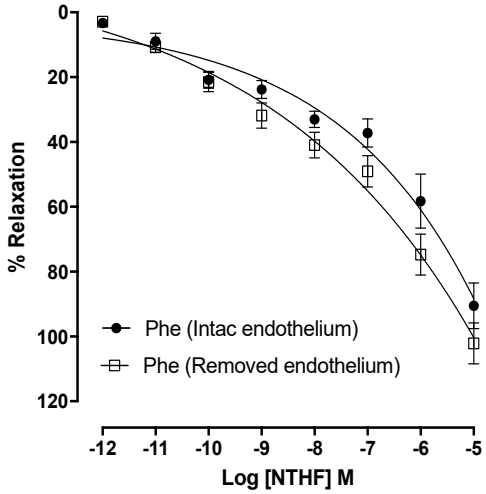

Figure 4. Vasodilator effect of NTHF in isolated rat mesenteric rings. Concentration–response curves showing the vasorelaxant effect induced by NTHF (10^{-12}–10^{-5} M) in mesenteric rings pre-contracted with phenylephrine (1 μM) in the presence (n = 6; •) or absence (n = 6; □) of functional endothelium. Values are expressed by mean ± S.E.M. The data were examined using Student's *t*-test.

After the addition of the last concentration of NTHF and following successive washes, the rings responded to a new stimulation induced by Phe (10 μM), with the contraction of a similar magnitude to that observed in the investigation of tissue viability.

3.4. Involvement of NO in the Vasorelaxant Response Induced by NTHF

To elucidate the mechanisms involved in the vasodilation induced by NTHF, the nitrate was added in the presence of $NO^{\bullet}$ scavengers, HDX and carboxy-PTIO. As shown in Figure 5, the vasorelaxant effect was significantly attenuated after the addition of both HDX (MR = 66 ± 9.2%, $p < 0.05$, and pD2 = 6.73 ± 0.28, $p < 0.05$; n = 5) and carboxy-PTIO (MR = 32 ± 6.2%, $p < 0.05$; and pD2 = 7.97 ± 0.37; n = 5), when compared to the control (MR = 100 ± 6.1%; and pD2 = 7.39 ± 0.15), suggesting that the vasodilation of the organic nitrate may be mediated by $NO^{\bullet}$.

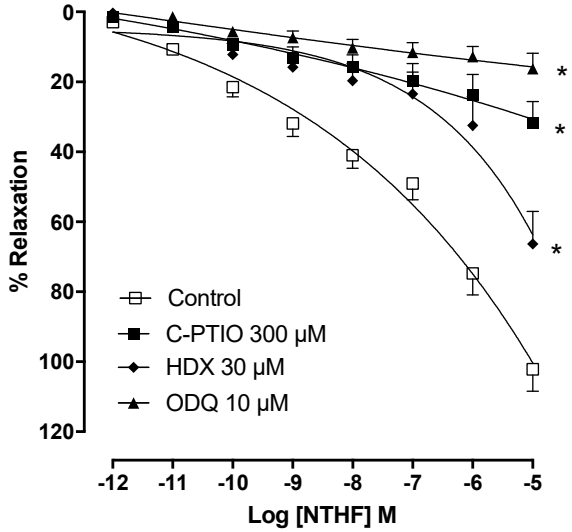

Figure 5. Concentration–response curves showing the involvement of NO/sGC/cGMP pathway in the vasorelaxant effect of NTHF. Relaxation induced by NTHF (10^{-12}–10^{-5} M) in the endothelium-denuded mesenteric artery rings pre-contracted with Phe (1 μM) in the absence (control; n = 6; □) or in the presence of C-PTIO (300 μM; n = 5; ■), HDX (30 μM; n = 5; ♦) or ODQ (10 μM; n = 5; ▲). Values are expressed by mean ± S.E.M. * $p < 0.05$ versus control. The data were examined using Student's *t*-test.

3.5. Soluble Guanylyl Cyclase Participates in the Vasodilation Induced by NTHF

Since sGC is the main target in the vascular smooth muscle cell, we investigated the participation of this enzyme using ODQ, a sGC inhibitor. The vasorelaxant response of NTHF was strongly inhibited when the mesenteric rings were pre-incubated with ODQ (MR = 22 ± 4.6%, $p < 0.05$; and pD2 = 9.10 ± 0.41, $p < 0.05$; n = 7), with a shift of the curve to the right and a reduction in the maximum effect when compared to the control (MR = 100 ± 6.1%; and pD2 = 7.39 ± 0.15; Figure 5). This result suggests that sGC is involved in the vasodilator response of the organic nitrate currently used in this study.

3.6. Vasodilatation Induced by NTHF Involves K^+ Channels Activation

Previously in this paper, we reported that the addition of high concentrations of K^+ attenuated the vasodilation induced by NTHF. It also suggests a contribution of K^+ channels in the response elicited by this NO donor. To better investigate this mechanism, we used some K^+ channel modulators.

In the presence of KCl (20 mM) [34], the vasorelaxant effect induced by NTHF was significantly attenuated (MR = 59 ± 9.5%, $p < 0.01$; pD2 = 6.92 ± 0.34; n = 8; Figure 6), corroborating that K^+ channels seem to participate in the vasorelaxant response of NTHF.

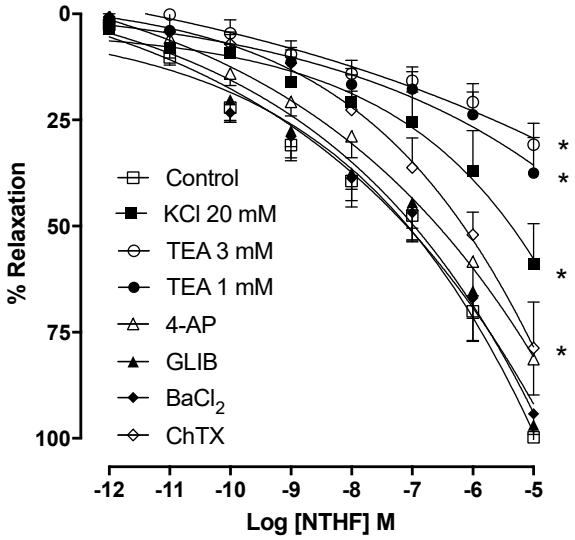

Figure 6. Concentration–response curves showing the participation of K^+ channels in the vasodilator effect induced by NTHF. Relaxation induced by NTHF (10^{-12}–10^{-5} M) in the endothelium-denuded mesenteric artery rings pre-contracted with Phe (1 μM) in the absence (control; n = 6; □) or in the presence of KCl (20 mM; n = 6; ■), TEA (3 mM; n = 6; ○), TEA (1 mM; n = 6; ●), 4-AP (1 mM; n = 6; △), GLI (10 μM; n = 6; ▲), ($BaCl_2$; 30 μM n = 6; ♦) or ChTX (100 nM; n = 6; ◇). Values are mean ± S.E.M. * $p < 0.05$ for versus control. The data were examined using Student's *t*-test.

A similar effect observed in the experiments carried out in the presence of the solution with KCl (20 mM) was also demonstrated after blocking the non-selective K^+ channel blocker with TEA (3 mM) (MR = 38 ± 8.3%, $p < 0.05$; pD2 = 7.36 ± 0.35; n = 7) compared to the control (MR = 100 ± 6.1%; pD2 = 7.39 ± 0.15) (Figure 6).

As shown in Figure 6 and Table 1, the presence of specific K^+ channel blockers, such as GLIB (10 μM) (MR = 97 ± 9.0%; and pD2 = 7.32 ± 0.24; n = 6), $BaCl_2$ (30 μM) (MR = 94 ± 4.9%; and pD2 = 7.36 ± 0.35; n = 5) and 4-AP (1 mM) (MR = 81 ± 8.5%; and pD2 = 7.41 ± 0.19; n = 8), produced no change in the vasorelaxant response produced by NTHF compared to the control curve (MR = 100 ± 6.1%; and pD2 = 7.39 ± 0.15). However, when we used TEA (1 mM), which in this concentration, selectively blocks the BK_{Ca} channels (MR = 31 ± 5.0%, $p < 0.05$; pD2 = 7.72 ± 0.30; n = 7) and ChTX, a well-known BK_{Ca} channel blocker (MR = 78 ± 10.2%, $p < 0.05$; pD2 = 6.96 ± 0.17, $p < 0.05$; n = 7), the vasorelaxant curve induced by NTHF was right-shifted, suggesting the involvement of BK_{Ca} channels in this response.

Table 1. Summary of the vascular reactivity results.

Protocol	ME	pD2
Intac endothelium	84 ± 5.3%	7.86 ± 0.20
Removed endothelium	100 ± 6.1%	7.39 ± 0.15
HDX	66 ± 9.2% *	6.73 ± 0.28
Carboxy-PTIO	32 ± 6.2% *	7.97 ± 0.37
ODQ	22 ± 4.6% *	9.10 ± 0.41 *
KCl (20 mM)	59 ± 9.5% *	6.92 ± 0.34
TEA (3 mM)	38 ± 8.3% *	7.36 ± 0.35
TEA (1 mM)	31 ± 5.0% *	7.72 ± 0.30
ChTX	78 ± 10.2% *	6.96 ± 0.17
GLIB	97 ± 9.0%	7.32 ± 0.24
$BaCl_2$	94 ± 4.9%	7.36 ± 0.35
4-AP	81 ± 8.5%	7.41 ± 0.19
NAC	89 ± 6.2%	8.32 ± 0.18 *

Values are expressed as mean ± S.E.M. * $p < 0.05$ vs. Control (removed endothelium). Student's *t*-test.

3.7. Reactive Oxygen Species Impair the Vasodilation Induced by NTHF

Here, we investigated whether oxidative stress impairs the vasodilation response induced by NTHF. When the rings were incubated with NAC (3 mM), a ROS scavenger (including NO^-), the NTHF concentration–response curve showed no difference in its effectiveness (MR = 89 ± 6.2%; n = 6). However, the vasodilator response of this organic nitrate was significantly potentiated (pD2 = 8.32 ± 0.18; $p < 0.05$) in the presence of this scavenger in comparison to the control (MR = 100 ± 6.1%; and pD2 = 7.39 ± 0.15; Figure 7), suggesting that ROS impair the relaxation of this organic nitrate.

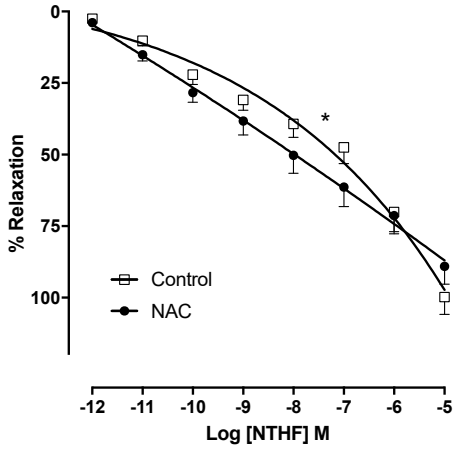

Figure 7. Concentration–response curves showing the participation of reactive oxygen species (ROS) in the vasorelaxant effect induced by NTHF. Concentration–response curves showing the vasorelaxant effect induced by NTHF (10^{-12}–10^{-5} M) in mesenteric rings pre-contracted with phenylephrine (1 μM) in the absence (control; n = 6; □) or in the presence of NAC (3 mM; n = 6; •). Values are expressed by mean ± S.E.M. * $p < 0.05$ versus control. The data were examined using Student's *t*-test.

3.8. Pre-Incubation with NTHF Does Not Induce Tolerance in the Nitrate-Vasodilator Effect

As nitrates used clinically are known to induce tolerance, we used a protocol in vitro to test this hypothesis with NTHF. Pre-incubation of NTHF (3; 10; 30 and 100 μM) did not change the sensitivity of the nitrate-vasodilator response (MR = 85.21 ± 4.9%; pD2 = 7.85 ± 0.18), (MR = 95 ± 6.7%, pD2 = 7.01 ± 0.17), (MR = 98.83 ± 5.2%; pD2 = 7.32 ± 0.18) and (MR = 88.38 ± 5.9%; pD2 = 7.81 ± 0.24 M), respectively, compared to the control without pre-treatment with NTHF (MR = 99.7 ± 6.1%; pD2 = 7.40 ± 0.15), as shown in Figure 8.

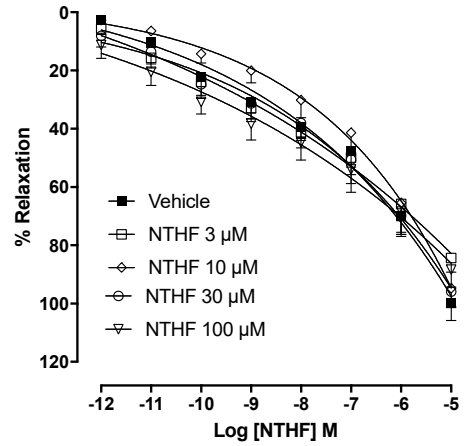

Figure 8. NTHF tolerance induced in vitro. Concentration–response curves showing the vasodilator effect induced by NTHF in mesenteric rings previously incubated with NTHF (3, 10, 30 and 100 μM) or vehicle for 1 h and pre-contracted with phenylephrine (1 μM). Values are expressed by mean ± S.E.M. The data were examined using Student's *t*-test.

4. Discussion

Organic nitrates are the oldest class of NO donors that have been clinically used [35,36]. They are classic vasodilators, including organic nitrates, organic nitrites and nitrite thiol esters, with organic nitrates being the most studied ones [37]. Here, we tested a new organic nitrate, recently synthesized, which is obtained from sugarcane agricultural waste, called NTHF.

The DAF-2DA probe is one of the most used fluorophores for NO detection, as it quantifies the cytoplasmic concentration of NO. The mode of action is divided into two stages: initially, this molecule has the ability to cross biological membranes; subsequently, it is hydrolyzed by plasma esterase, forming DAF-2, which in the presence of NO is converted into triazole fluoriscein (DAF-2T), this being the fluorescent form. Under these conditions, the molecule can be excited and emits green light, making it as a good tool for analyzing NO production from NO donors [38]. In VSMCs from rat aorta, NTHF promoted an increase in fluorescence, attributed to the increase in NO levels. This increase was also observed in the presence of another NO donor, the spontaneous donor (SNP) as well as a donor that requires enzymatic bioconversion (GTN). Similar findings were demonstrated in the presence of nitrates with GTN and another nitrate used by us, 2-nitrate-1,3-dibuthoxypropan (NDBP) in CMLV of rat aorta [32]. In this last study, our group used two different concentrations of GTN (10 μM and 100 μM). The NO release from the major concentration was bigger than the NO release from the new nitrate; however, 10 μM of GTN did not show any difference.

Previously, we revealed that other experimental organic nitrates, such as the (Z)-ethyl 12- nitrooxy-octadec-9-enoate (NCOE), elicited a growth in the DAF-induced fluorescence in VSMC, indicating an augment in NO concentrations. This effect was also stronger than in SNP [31]. It was similar to the effect here presented by NTHF, where we can see a remarkable increase in NO levels compared to SNP or NTG. It is important to highlight that the concentration of nitrates used previously (NCOE) [31] as well as the one used in the present research was higher (100 μM) than SNP and NTG. All together, these results show that different nitrates are capable to promote NO production and also have distinct ways to induce the release (enzymatic or spontaneously).

In our experiments, we showed that NTHF induced dose-dependent hypotension in unanesthetized rats. The hypotensive effect elicited by the last three doses of NTHF (30, 40 and 50 mg/kg) was quick and lasted around 20 s, while the first two doses (10 and 20 mg/kg) presented a low impact on blood pressure reduction. The rapid response found after NTHF administration could be explained by the fleeting effect of the NO. In addition, the vehicle did not alter the blood pressure values. Similar effects to the NTHF have been reported by us and others using different NO donors in normotensive rats, such as the NCOE [31] and the cis-$[Ru(bpy)_2(py)(NO_2)](PF_6)$ (RuBPY) [39] as well as in hypertensive animals, for instance, NDBP [40,41] and the nitrosyl ruthenium complex $[Ru(terpy)(bdq)NO^+]3^+$ (TERPY) [42,43].

NO promotes positive and negative inotropic effects in low and high doses, respectively [44–46]. It happens due to the increased affinity to the heart muscle in the presence of high NO concentrations [47]. This fact corroborates with our data, since the bradycardic effect induced by NTHF markedly occurs at the highest doses. Klimaschewski, et al. [48] demonstrated that NOS is present in ganglion cells and cardiac fibers, which innervate the sinoatrial and atrioventricular node in the myocardium and in neurons close to coronary vessels. This finding suggests that NO is an important regulator of myocardial cell function and promotes a negative chronotropic effect through PKG activation [49]. Bradycardia is mainly influenced by ACh discharges from the parasympathetic vagus nerve, which can be modulated by the activation of the NOS-sGC-PKG pathway and also by NO donors [50]. In addition, findings have showed that the overexpression of eNOS in different areas of the brain, for instance, the rostral ventrolateral medulla and nucleus of the solitary tract, induced hypotension and bradycardia associated with sympathoinhibition [51–53]. Furthermore, more recent studies have demonstrated that NO derived from nNOS in the hypothalamic paraventricular nucleus possess a key role by decreasing renal sympathetic

activity and blood pressure in rats [54,55]. Clearly, the NO released from the donors could promote a reduction in the arterial pressure by reducing peripheral vascular resistance.

Due to the importance of the factors released by the endothelium in controlling the vascular tone, experiments were carried out in mesenteric rings without an endothelium and the vasorelaxant effect promoted by NTHF was not modified; however, it showed greater efficacy, suggesting that relaxing factors derived from the endothelium are not involved in the vasodilator response of the organic nitrate. A similar response was found when the endothelium was removed in the presence of Cis-$[Ru(bpy)_2ImN(NO)](PF_6)_3$ (named FOR 0811) and the concentration–response curve to FOR 0811 was not altered [56]. Testing another new NO donor, called 1,3-bis (hexyloxy) propan-2-yl nitrate (NDHP), the authors also reported an independence of the endothelium in the vasorelaxant effect induced by NDHP; however, they found that in the mesenteric rings without an endothelium, the maximum response was higher compared to the curves with an intact endothelium [57]. This effect was also observed in the current paper. On the other hand, TERPY, a NO-donating metallodrug, induced a decrease in the vasorelaxant potency under the same experimental conditions, indicating a relevant role of the endothelial layer in the mechanism of the action of the TERPY [58]. These effects suggest that the participation of the endothelium depends on the compound (NO donor), presenting different responses. Based on the effect promoted by NTHF, all subsequent experimental protocols here were performed in the absence of a vascular endothelium.

Classically, NO can induce a vasodilator effect, in most instances, by the subsequently generated second messenger cGMP after its binding into sGC [59–63]. Therefore, we tested the involvement of the NO/sGC/cGMP pathway in the effect induced by NTHF using different pharmacological tools such as HDX, used to investigate physiological processes mediated by $NO^{\bullet}$ [56,64], functioning through the binding of its cobalt atom to NO, converting itself to nitrosocobalamin and inactivating this radical [65], carboxy-PTIO, which is a stable radical that oxidizes NO to generate nitrogen dioxide (NO_2) and 2-phenyll-4,4,5,5-tetramethy-limidazoline-1-oxyl (PTI) [66]. This radical is a scavenger of the radical form of NO [67], blocking the response to exogenous $NO^{\bullet}$ [68,69]. ODQ is a pharmacological tool that competitively binds to the same NO binding site in sGC, oxidizing the Fe^{2+} of the heme group and promoting the irreversible inhibition of the enzyme [51,70,71]. In the presence of all three pharmacological agents, the maximum response induced by NTHF was attenuated. Thus, our data clearly demonstrate that NTHF exerts its vasodilator response through the release of NO, probably in the radical form, which binds into sGC, generating cGMP in the vascular smooth muscle.

The increase in cGMP levels, generated by sGC activation, modulates several intracellular processes [72] via the activation of their downstream effectors such as PKG [73] and ion channels operated by cyclic nucleotides [74]. One of the main consequences of PKG activation in VSMC is vasorelaxation, which is mediated by the phosphorylation of proteins that regulate intracellular Ca^{2+} levels, the sensitivity of the contractile machinery and the increased activity of K^+ channels [75].

The addition of Tyrode's solution containing KCl (20 mM) and pre-contracted with Phe induced a shift of the curve to the right and a reduction in the maximum effect. It suggests that the vasorelaxant effect of this organic nitrate probably involves the participation of K^+ channels since relevant studies have shown that the vasodilator drugs whose mechanisms are dependent on K^+ channels exhibit a loss of their effects when exposed to solutions with a high concentration of K^+. An increase in extracellular K^+ attenuates the electrochemical gradient of this ion across the membrane, making the activation mechanism of these channels ineffective [76,77].

To reinforce this hypothesis, TEA (3 mM) was used, which, at this concentration, behaves as a non-selective blocker of K^+ channels [78]. The NTHF response was attenuated, with a reduction in the maximum effect, corroborating the involvement of K^+ channels in the vasodilator effect of this nitrate.

Previous studies on VSMC have provided evidence for the contribution of K^+ channels in the pathway of NO-induced relaxation. In pulmonary artery smooth muscle cells, a selective stimulation of PKG was found to increase K^+ currents [79]. It is also assumed that NO can directly activate K^+ channels independently of cGMP [72]. In addition, findings from patch-clamp trials suggest that K^+ channels, such as the BK_{Ca} channels, may be activated via the NO-cGMP pathway [80,81] and that these types of channels regulate the vasorelaxant effect to both exogenous nitro vasodilators and an endogenous receptor-mediated NO release in isolated arteries [60,76,82].

We next assessed which subtypes of K^+ channels identified in VSMCs would be participating in this nitrate vasodilator response. For this purpose, selective blockers were used for each channel (BK_{Ca}, K_V, K_{ATP} and K_{IR}). To evaluate the participation of other K^+ channels found in vascular smooth muscle, selective blockers were used for the other channels: 4-AP, a K_V blocker, GLIB, a blocker of K_{ATP} channels and $BaCl_2$, a selective blocker for K_{IR}. After the individual pre-incubation of these selective blockers, the vasorelaxation promoted by NTHF was not modified, suggesting that the K_V, K_{ATP} and K_{IR} channels probably are not involved in the vasodilator response of the nitrate used in the current study.

It is well reported that TEA, in concentrations lower than 1 mM, selectively blocks BK_{Ca} [34,83]. After the pre-incubation of the mesenteric rings with this blocker, the NTHF vasodilator response was attenuated similarly to that observed with the major concentration of TEA, suggesting that the K^+ channels type involved in the NTHF vasorelaxant response is BK_{Ca}.

ChTX is a non-specific BK_{Ca} channel blocker operating simultaneously on the IK_{Ca} channels and slowly disabling voltage-dependent K^+ channels ($K_{v1.3}$) [84,85]. Experiments performed after the incubation of ChTX demonstrated that the relaxant effect produced by NTHF was reduced, corroborating the previous results using other pharmacological tools. Hence, these results support the finding that BK_{Ca} is the K^+ channel subtype involved in the vasorelaxant effect induced by NTHF.

To clarify if the ROS production would induce NO sequestration, an experimental protocol was developed using NAC, a thiol, a pharmacological precursor of L-cysteine and exogenous donor of sulfhydryl groups, which acts by sequestering ROS [86]. This thiol potentiated the vasorelaxant response of NTHF, corroborating with studies that showed that NAC may potentiate the effects of nitrates [87]. NO also can react with thiols in vivo to form S-nitrosothiols [88]. It has also been suggested that S-nitrosothiols may be considered as a storage form for intravascular NO, as they are more stable than NO and are thought to act as NO donor molecules in the circulation [89]. So, the effect of NTHF in the presence of NAC may be related to the formation of S-nitrosothiols [90,91], which protects NO from free radical degradation, as well as to the improvement of NO bioavailability due to NAC-mediated ROS scavenging.

Although organic nitrates are excellent agents for the treatment of cardiovascular diseases, their use is limited by the fact that most of these compounds develop vascular tolerance [92,93]. Our results suggest that this study's maximum concentration of NTHF does not develop vascular tolerance after prolonged exposure to the cumulative concentrations of NTHF.

During the investigation of the vascular tolerance, NTHF was incubated for 60 min and after cumulative concentrations of the nitrate, no decrease in the vasorelaxant response was observed. Previous studies with nitrates such as GTN, PETN and pentaerythrityl trinitrate (PEtriN) have already shown vascular tolerance with 30 min exposure using 300 μM (in bolus) in the porcine pulmonary artery [94]. Additional studies could be carried out to evaluate another possibility, which would be to study the effects of NTHF on vessels exposed to a nitrate that induces vascular tolerance and then observe whether it would still be able to induce tolerance. Koenig et al. (2007) demonstrated the strong attenuation of the vasodilator response after the repeated administration of GTN, which was also seen in studies with isolated porcine arteries. Furthermore, studies demonstrated that the

profile of in vitro tolerance for PETN and PEtriN was similar to GTN [95]. In addition, the dinitrates and mononitrates developed little or no tolerance in vitro. It means that the vascular tolerance increases with the number of nitrate groups in the molecule and also with the potency of the nitrate; however, it is not linked with the vasodilator properties [94]. The fact that NTHF has only one nitrate group may be a reasonable explanation for the lack of vascular tolerance found here. This result shows great importance for this study, based on the fact that the most organic nitrates in current use induce tolerance [96,97].

5. Conclusions

In conclusion, all results together indicate that NTHF promotes an endothelium-independent vasodilator effect in the superior mesenteric artery rings of rats. In addition, this effect involves $NO^{\bullet}$ production following the possible activation of the sGC with the participation of the large-conductance Ca^{2+}-sensitive K^+ channels—BK_{Ca}. Furthermore, the organic nitrate studied in this paper did not develop vascular tolerance under the experimental conditions presented here (Figure 9). Future studies are needed to reveal the molecular mechanism involved in the vasodilatory effect of NTHF, such as the evaluation of K^+ currents in dispersed mesenteric artery myocytes and the confirmation of the subtype of the K^+ channel involved, as well as the measurement of the cGMP levels. In addition, we intend to evaluate the effect of NTHF on myocardial infarction, one of the main cardiovascular diseases treated with organic nitrates.

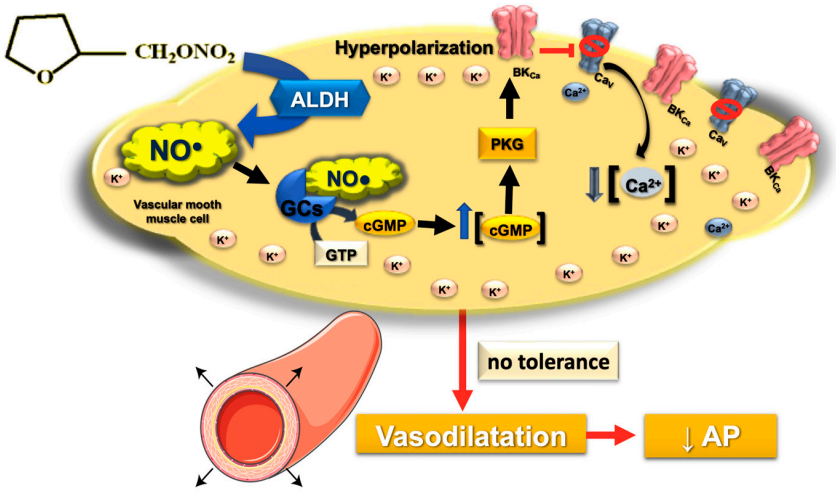

Figure 9. NTHF-induced hypotension and vasorelaxant effect. The $NO^{\bullet}$, which is released from NTHF molecule, binds into sGC and activates the $NO^{\bullet}$/cGC/PKG pathway and causes subsequent activation of large-conductance Ca^{2+}ensitive K^+ channels (BK_{Ca2+}). Furthermore, under the experimental conditions performed here, NTHF did not develop vascular tolerance after prolonged exposure to the cumulative concentrations of the organic nitrate.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Federal University of Paraiba CEUA/UFPB (protocol # 0310/09 and 0207/12).

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References

1. Lee, J.; Bae, E.H.; Ma, S.K.; Kim, S.W. Altered nitric oxide system in cardiovascular and renal diseases. *Chonnam Med. J.* **2016**, *52*, 81–90. [CrossRef] [PubMed]
2. Katusic, Z.S.; Austin, S.A. Endothelial nitric oxide: Protector of a healthy mind. *Eur. Heart J.* **2014**, *35*, 888–894. [CrossRef] [PubMed]
3. Chen, G.; Zhang, L.; Van Schepdael, A.; Wang, X. Recent Advances in Activation of Endothelial Nitric Oxide Synthase by Natural Products: An Effects and Mechanisms Review. *Food Rev. Int.* **2023**, *40*, 260–275. [CrossRef]
4. Lei, J.; Vodovotz, Y.; Tzeng, E.; Billiar, T.R. Nitric oxide, a protective molecule in the cardiovascular system. *Nitric Oxide* **2013**, *35*, 175–185. [CrossRef]
5. Moncada, S.; Higgs, E.A. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **1995**, *9*, 1319–1330. [CrossRef]
6. Tousoulis, D.; Kampoli, A.M.; Tentolouris, C.; Papageorgiou, N.; Stefanadis, C. The role of nitric oxide on endothelial function. *Curr. Vasc. Pharmacol.* **2012**, *10*, 4–18. [CrossRef]
7. Cyr, A.R.; Huckaby, L.V.; Shiva, S.S.; Zuckerbraun, B.S. Nitric Oxide and Endothelial Dysfunction. *Crit. Care Clin.* **2020**, *36*, 307–321. [CrossRef]
8. Li, H.; Förstermann, U. Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* **2000**, *190*, 244–254. [CrossRef]
9. Wever, R.M.; van Dam, T.; van Rijn, H.J.; de Groot, F.; Rabelink, T.J. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 340–344. [CrossRef]
10. Chalupsky, K.; Cai, H. Endothelial dihydrofolate reductase: Critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 9056–9061. [CrossRef]
11. Siu, K.L.; Lotz, C.; Ping, P.; Cai, H. Netrin-1 abrogates ischemia/reperfusion-induced cardiac mitochondrial dysfunction via nitric oxide-dependent attenuation of NOX4 activation and recoupling of NOS. *J. Mol. Cell. Cardiol.* **2015**, *78*, 174–185. [CrossRef] [PubMed]
12. Zhang, Y.; Murugesan, P.; Huang, K.; Cai, H. NADPH oxidases and oxidase crosstalk in cardiovascular diseases: Novel therapeutic targets. *Nat. Rev. Cardiol.* **2020**, *17*, 170–194. [CrossRef] [PubMed]
13. Favero, G.; Paganelli, C.; Buffoli, B.; Rodella, L.F.; Rezzani, R. Endothelium and its alterations in cardiovascular diseases: Life style intervention. *Biomed. Res. Int.* **2014**, *2014*, 801896. [CrossRef] [PubMed]
14. Rubbo, H.; Radi, R.; Trujillo, M.; Telleri, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B.A. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **1994**, *269*, 26066–26075. [CrossRef] [PubMed]
15. Nappi, F.; Fiore, A.; Masiglat, J.; Cavuoti, T.; Romandini, M.; Nappi, P.; Avtaar Singh, S.S.; Couetil, J.-P. Endothelium-Derived Relaxing Factors and Endothelial Function: A Systematic Review. *Biomedicines* **2022**, *10*, 2884. [CrossRef]
16. Miller, M.; Megson, I. Recent developments in nitric oxide donor drugs. *Br. J. Pharmacol.* **2007**, *151*, 305–321. [CrossRef]
17. Paulo, M.; Costa, D.E.; Bonaventura, D.; Lunardi, C.N.; Bendhack, L.M. Nitric oxide donors as potential drugs for the treatment of vascular diseases due to endothelium dysfunction. *Curr. Pharm. Des.* **2020**, *26*, 3748–3759. [CrossRef]
18. Ignarro, L.J.; Napoli, C.; Loscalzo, J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: An overview. *Circ. Res.* **2002**, *90*, 21–28. [CrossRef]
19. Daiber, A.; Xia, N.; Steven, S.; Oelze, M.; Hanf, A.; Kröller-Schön, S.; Münzel, T.; Li, H. New therapeutic implications of endothelial nitric oxide synthase (eNOS) function/dysfunction in cardiovascular disease. *Int. J. Mol. Sci.* **2019**, *20*, 187. [CrossRef]
20. Johal, T.; Lees, C.C.; Everett, T.R.; Wilkinson, I.B. The nitric oxide pathway and possible therapeutic options in pre-eclampsia. *Br. J. Clin. Pharmacol.* **2014**, *78*, 244–257. [CrossRef]
21. Naghavi, N.; de Mel, A.; Alavijeh, O.S.; Cousins, B.G.; Seifalian, A.M. Nitric oxide donors for cardiovascular implant applications. *Small* **2013**, *9*, 22–35. [CrossRef] [PubMed]
22. Hollas, M.A.; Aissa, M.B.; Lee, S.H.; Gordon-Blake, J.M.; Thatcher, G.R. Pharmacological manipulation of cGMP and NO/cGMP in CNS drug discovery. *Nitric Oxide* **2019**, *82*, 59–74. [CrossRef] [PubMed]
23. Marini, E.; Giorgis, M.; Leporati, M.; Rolando, B.; Chegaev, K.; Lazzarato, L.; Bertinaria, M.; Vincenti, M.; Di Stilo, A. Multitarget Antioxidant NO-Donor Organic Nitrates: A Novel Approach to Overcome Nitrates Tolerance, an Ex Vivo Study. *Antioxidants* **2022**, *11*, 166. [CrossRef] [PubMed]

24. Rudyk, O.; Prysyazhna, O.; Burgoyne, J.R.; Eaton, P. Nitroglycerin fails to lower blood pressure in redox-dead Cys42Ser PKG1α knock-in mouse. *Circulation* **2012**, *126*, 287–295. [CrossRef] [PubMed]
25. Zhu, D.; Hou, J.; Qian, M.; Jin, D.; Hao, T.; Pan, Y.; Wang, H.; Wu, S.; Liu, S.; Wang, F. Nitrate-functionalized patch confers cardioprotection and improves heart repair after myocardial infarction via local nitric oxide delivery. *Nat. Commun.* **2021**, *12*, 4501. [CrossRef]
26. Gori, T.; Mak, S.S.; Kelly, S.; Parker, J.D. Evidence supporting abnormalities in nitric oxide synthase function induced by nitroglycerin in humans. *J. Am. Coll. Cardiol.* **2001**, *38*, 1096–1101. [CrossRef]
27. Münzel, T.; Daiber, A.; Gori, T. More answers to the still unresolved question of nitrate tolerance. *Eur. Heart J.* **2013**, *34*, 2666–2673. [CrossRef]
28. Daiber, A.; Oelze, M.; Coldewey, M.; Bachschmid, M.; Wenzel, P.; Sydow, K.; Wendt, M.; Kleschyov, A.L.; Stalleicken, D.; Ullrich, V. Oxidative stress and mitochondrial aldehyde dehydrogenase activity: A comparison of pentaerythritol tetranitrate with other organic nitrates. *Mol. Pharmacol.* **2004**, *66*, 1372–1382. [CrossRef]
29. Sayed, N.; Kim, D.D.; Fioramonti, X.; Iwahashi, T.; Durán, W.N.; Beuve, A. Nitroglycerin-induced S-nitrosylation and desensitization of soluble guanylyl cyclase contribute to nitrate tolerance. *Circ. Res.* **2008**, *103*, 606–614. [CrossRef]
30. Gori, T.; Burstein, J.M.; Ahmed, S.; Miner, S.E.; Al-Hesayen, A.; Kelly, S.; Parker, J.D. Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: A human in vivo study. *Circulation* **2001**, *104*, 1119–1123. [CrossRef]
31. Machado, N.T.; Maciel, P.M.; Alustau, M.C.; Queiroz, T.M.; Furtado, F.F.; Assis, V.L.; Veras, R.C.; Araújo, I.G.; Athayde-Filho, P.F.; Medeiros, I.A. Nitric oxide as a target for the hypotensive and vasorelaxing effects induced by (Z)-ethyl 12-nitrooxy-octadec-9-enoate in rats. *Eur. J. Pharm. Sci.* **2014**, *62*, 317–325. [CrossRef] [PubMed]
32. França-Silva, M.S.; Luciano, M.N.; Ribeiro, T.P.; Silva, J.S.; Santos, A.F.; França, K.C.; Nakao, L.S.; Athayde-Filho, P.F.; Braga, V.A.; Medeiros, I.A. The 2-nitrate-1, 3-dibuthoxypropan, a new nitric oxide donor, induces vasorelaxation in mesenteric arteries of the rat. *Eur. J. Pharmacol.* **2012**, *690*, 170–175. [CrossRef] [PubMed]
33. Queiroz, T.M.; Machado, N.T.; Furtado, F.F.; Oliveira-Filho, A.A.; Alustau, M.C.; Figueiredo, C.S.; Miranda, G.E.; Barbosa-Filho, J.M.; Braga, V.A.; Medeiros, I.A. Vasorelaxation, induced by *Dictyota pulchella* (Dictyotaceae), a brown alga, is mediated via inhibition of calcium influx in rats. *Mar. Drugs* **2011**, *9*, 2075–2088. [CrossRef] [PubMed]
34. Silva, D.F.; Araújo, I.G.; Albuquerque, J.G.; Porto, D.L.; Dias, K.L.; Cavalcante, K.V.; Veras, R.C.; Nunes, X.P.; Barbosa-Filho, J.M.; Araújo, D.A.; et al. Rotundifolone-induced relaxation is mediated by $BK_{(Ca)}$ channel activation and $Ca_{(v)}$ channel inactivation. *Basic Clin. Pharmacol. Toxicol.* **2011**, *109*, 465–475. [CrossRef]
35. Wang, P.G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A.J. Nitric oxide donors: Chemical activities and biological applications. *Chem. Rev.* **2002**, *102*, 1091–1134. [CrossRef]
36. Franca-Silva, M.S.; Balarini, C.M.; Cruz, J.C.; Khan, B.A.; Rampelotto, P.H.; Braga, V.A. Organic nitrates: Past, present and future. *Molecules* **2014**, *19*, 15314–15323. [CrossRef]
37. Yang, Y.; Huang, Z.; Li, L.L. Advanced nitric oxide donors: Chemical structure of NO drugs, NO nanomedicines and biomedical applications. *Nanoscale* **2021**, *13*, 444–459. [CrossRef]
38. Bryan, N.S.; Grisham, M.B. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med.* **2007**, *43*, 645–657. [CrossRef]
39. Pereira, A.C.; Araujo, A.V.; Paulo, M.; Andrade, F.A.; Silva, B.R.; Vercesi, J.A.; da Silva, R.S.; Bendhack, L.M. Hypotensive effect and vascular relaxation in different arteries induced by the nitric oxide donor RuBPY. *Nitric Oxide* **2017**, *62*, 11–16. [CrossRef]
40. França-Silva, M.S.; Monteiro, M.M.; Queiroz, T.M.; Santos, A.F.; Athayde-Filho, P.F.; Braga, V.A. The new nitric oxide donor 2-nitrate-1,3-dibuthoxypropan alters autonomic function in spontaneously hypertensive rats. *Auton. Neurosci.* **2012**, *171*, 28–35. [CrossRef]
41. Queiroz, T.M.; Mendes-Júnior, L.G.; Guimarães, D.D.; França-Silva, M.S.; Nalivaiko, E.; Braga, V.A. Cardiorespiratory effects induced by 2-nitrate-1,3-dibuthoxypropan are reduced by nitric oxide scavenger in rats. *Auton. Neurosci.* **2014**, *181*, 31–36. [CrossRef] [PubMed]
42. Munhoz, F.C.; Potje, S.R.; Pereira, A.C.; Daruge, M.G.; da Silva, R.S.; Bendhack, L.M.; Antoniali, C. Hypotensive and vasorelaxing effects of the new NO-donor $[Ru(terpy)(bdq)NO^{(+)}]^{(3+)}$ in spontaneously hypertensive rats. *Nitric Oxide* **2012**, *26*, 111–117. [CrossRef] [PubMed]
43. Potje, S.R.; Hildebrand, M.C.; Munhoz, F.C.; Troiano, J.A.; Pereira, A.A.; Nakamune, A.C.; da Silva, R.S.; Bendhack, L.M.; Antoniali, C. The hypotensive effect of the ruthenium complex $[Ru(terpy)(bdq)NO]^{(3)(+)}$ is higher in male than in female spontaneously hypertensive rats (SHR). *Naunyn Schmiedebergs Arch. Pharmacol.* **2014**, *387*, 1045–1051. [CrossRef]
44. Mohan, P.; Sys, S.U.; Brutsaert, D.L. Positive inotropic effect of nitric oxide in myocardium. *Int. J. Cardiol.* **1995**, *50*, 233–237. [CrossRef]
45. Wyeth, R.P.; Temma, K.; Seifen, E.; Kennedy, R.H. Negative inotropic actions of nitric oxide require high doses in rat cardiac muscle. *Pflüg. Arch.* **1996**, *432*, 678–684. [CrossRef] [PubMed]
46. Vila-Petroff, M.G.; Younes, A.; Egan, J.; Lakatta, E.G.; Sollott, S.J. Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ. Res.* **1999**, *84*, 1020–1031. [CrossRef] [PubMed]
47. Brunner, F.; Andrew, P.; Wölkart, G.; Zechner, R.; Mayer, B. Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Circulation* **2001**, *104*, 3097–3102. [CrossRef]
48. Klimaschewski, L.; Kummer, W.; Mayer, B.; Couraud, J.Y.; Preissler, U.; Philippin, B.; Heym, C. Nitric oxide synthase in cardiac nerve fibers and neurons of rat and guinea pig heart. *Circ. Res.* **1992**, *71*, 1533–1537. [CrossRef]

49. Shah, A.M.; Spurgeon, H.A.; Sollott, S.J.; Talo, A.; Lakatta, E.G. 8-bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes. *Circ. Res.* **1994**, *74*, 970–978. [CrossRef]
50. Herring, N.; Paterson, D.J. Neuromodulators of peripheral cardiac sympatho-vagal balance. *Exp. Physiol.* **2009**, *94*, 46–53. [CrossRef]
51. Garthwaite, J. NO as a multimodal transmitter in the brain: Discovery and current status. *Br. J. Pharmacol.* **2019**, *176*, 197–211. [CrossRef] [PubMed]
52. Sakai, K.; Hirooka, Y.; Matsuo, I.; Eshima, K.; Shigematsu, H.; Shimokawa, H.; Takeshita, A. Overexpression of eNOS in NTS causes hypotension and bradycardia in vivo. *Hypertension* **2000**, *36*, 1023–1028. [CrossRef] [PubMed]
53. Kishi, T.; Hirooka, Y.; Sakai, K.; Shigematsu, H.; Shimokawa, H.; Takeshita, A. Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release. *Hypertension* **2001**, *38*, 896–901. [CrossRef] [PubMed]
54. Hirooka, Y. Sympathetic Activation in Hypertension: Importance of the Central Nervous System. *Am. J. Hypertens.* **2020**, *33*, 914–926. [CrossRef]
55. McBryde, F.D.; Liu, B.H.; Roloff, E.V.; Kasparov, S.; Paton, J.F.R. Hypothalamic paraventricular nucleus neuronal nitric oxide synthase activity is a major determinant of renal sympathetic discharge in conscious Wistar rats. *Exp. Physiol.* **2018**, *103*, 419–428. [CrossRef]
56. Costa, P.P.C.; Campos, R.; Cabral, P.H.B.; Gomes, V.M.; Santos, C.F.; Waller, S.B.; de Sousa, E.H.S.; Lopes, L.G.F.; Fonteles, M.C.; do Nascimento, N.R.F. Antihypertensive potential of cis-[Ru(bpy)2(ImN)(NO)](3+), a ruthenium-based nitric oxide donor. *Res. Vet. Sci.* **2020**, *130*, 153–160. [CrossRef]
57. Paulo, L.L.; Cruz, J.C.; Zhuge, Z.; Carvalho-Galvao, A.; Brandao, M.C.R.; Diniz, T.F.; Haworth, S.M.; Athayde-Filho, P.F.; Lemos, V.S.; Lundberg, J.O.; et al. The novel organic mononitrate NDHP attenuates hypertension and endothelial dysfunction in hypertensive rats. *Redox Biol.* **2018**, *15*, 182–191. [CrossRef]
58. Bonaventura, D.; Lunardi, C.N.; Rodrigues, G.J.; Neto, M.A.; Vercesi, J.A.; de Lima, R.G.; da Silva, R.S.; Bendhack, L.M. Endothelium negatively modulates the vascular relaxation induced by nitric oxide donor, due to uncoupling NO synthase. *J. Inorg. Biochem.* **2009**, *103*, 1366–1374. [CrossRef]
59. Waldman, S.A.; Murad, F. Biochemical mechanisms underlying vascular smooth muscle relaxation: The guanylate cyclase-cyclic GMP system. *J. Cardiovasc. Pharmacol.* **1988**, *12* (Suppl. S5), S115–S118. [CrossRef]
60. Sand, A.; Andersson, E.; Fried, G. Nitric oxide donors mediate vasodilation in human placental arteries partly through a direct effect on potassium channels. *Placenta* **2006**, *27*, 181–190. [CrossRef]
61. Fernhoff, N.B.; Derbyshire, E.R.; Marletta, M.A. A nitric oxide/cysteine interaction mediates the activation of soluble guanylate cyclase. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 21602–21607. [CrossRef]
62. Gibb, B.J.; Wykes, V.; Garthwaite, J. Properties of NO-activated guanylyl cyclases expressed in cells. *Br. J. Pharmacol.* **2003**, *139*, 1032–1040. [CrossRef] [PubMed]
63. Da Silva, G.M.; da Silva, M.C.; Nascimento, D.V.G.; Lima Silva, E.M.; Gouvêa, F.F.F.; de França Lopes, L.G.; Araújo, A.V.; Ferraz Pereira, K.N.; de Queiroz, T.M. Nitric Oxide as a Central Molecule in Hypertension: Focus on the Vasorelaxant Activity of New Nitric Oxide Donors. *Biology* **2021**, *10*, 1041. [CrossRef] [PubMed]
64. Rajanayagam, M.A.; Li, C.G.; Rand, M.J. Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br. J. Pharmacol.* **1993**, *108*, 3–5. [CrossRef] [PubMed]
65. Rand, M.J.; Li, C.G. Discrimination by the NO-trapping agent, carboxy-PTIO, between NO and the nitrergic transmitter but not between NO and EDRF. *Br. J. Pharmacol.* **1995**, *116*, 1906–1910. [CrossRef]
66. Goldstein, S.; Russo, A.; Samuni, A. Reactions of PTIO and carboxy-PTIO with *NO, *NO_2, and O_2-*. *J. Biol. Chem.* **2003**, *278*, 50949–50955. [CrossRef]
67. Akaike, T.; Yoshida, M.; Miyamoto, Y.; Sato, K.; Kohno, M.; Sasamoto, K.; Miyazaki, K.; Ueda, S.; Maeda, H. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/.NO through a radical reaction. *Biochemistry* **1993**, *32*, 827–832. [CrossRef]
68. Hobbs, A.J.; Tucker, J.F.; Gibson, A. Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: Role of superoxide anions. *Br. J. Pharmacol.* **1991**, *104*, 645–650. [CrossRef]
69. Dantas, B.P.; Ribeiro, T.P.; Assis, V.L.; Furtado, F.F.; Assis, K.S.; Alves, J.S.; Silva, T.M.; Camara, C.A.; França-Silva, M.S.; Veras, R.C.; et al. Vasorelaxation induced by a new naphthoquinone-oxime is mediated by NO-sGC-cGMP pathway. *Molecules* **2014**, *19*, 9773–9785. [CrossRef]
70. Garthwaite, J.; Southam, E.; Boulton, C.L.; Nielsen, E.B.; Schmidt, K.; Mayer, B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one. *Mol. Pharmacol.* **1995**, *48*, 184–188.
71. Schrammel, A.; Behrends, S.; Schmidt, K.; Koesling, D.; Mayer, B. Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.* **1996**, *50*, 1–5. [PubMed]
72. Bolotina, V.M.; Najibi, S.; Palacino, J.J.; Pagano, P.J.; Cohen, R.A. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **1994**, *368*, 850–853. [CrossRef] [PubMed]
73. Robertson, B.E.; Schubert, R.; Hescheler, J.; Nelson, M.T. cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.* **1993**, *265*, C299–C303. [CrossRef] [PubMed]
74. Zaccolo, M.; Movsesian, M.A. cAMP and cGMP signaling cross-talk: Role of phosphodiesterases and implications for cardiac pathophysiology. *Circ. Res.* **2007**, *100*, 1569–1578. [CrossRef]

75. Takahashi, S.; Lin, H.; Geshi, N.; Mori, Y.; Kawarabayashi, Y.; Takami, N.; Mori, M.X.; Honda, A.; Inoue, R. Nitric oxide-cGMP-protein kinase G pathway negatively regulates vascular transient receptor potential channel TRPC6. *J. Physiol.* **2008**, *586*, 4209–4223. [CrossRef]
76. Khan, S.A.; Higdon, N.R.; Meisheri, K.D. Coronary vasorelaxation by nitroglycerin: Involvement of plasmalemmal calcium-activated K+ channels and intracellular Ca++ stores. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 838–846.
77. Menezes, I.A.; Moreira, I.J.; Carvalho, A.A.; Antoniolli, A.R.; Santos, M.R. Cardiovascular effects of the aqueous extract from Caesalpinia ferrea: Involvement of ATP-sensitive potassium channels. *Vasc. Pharmacol.* **2007**, *47*, 41–47. [CrossRef]
78. Wang, S.P.; Zang, W.J.; Kong, S.S.; Yu, X.J.; Sun, L.; Zhao, X.F.; Wang, S.X.; Zheng, X.H. Vasorelaxant effect of isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate, a novel metabolite from *Salvia miltiorrhiza*, on isolated rat mesenteric artery. *Eur. J. Pharmacol.* **2008**, *579*, 283–288. [CrossRef]
79. Hampl, V.; Huang, J.M.; Weir, E.K.; Archer, S.L. Activation of the cGMP-dependent protein kinase mimics the stimulatory effect of nitric oxide and cGMP on calcium-gated potassium channels. *Physiol. Res.* **1995**, *44*, 39–44.
80. Liu, B.; Yang, J.; Wen, Q.; Li, Y. Isoliquiritigenin, a flavonoid from licorice, relaxes guinea-pig tracheal smooth muscle in vitro and in vivo: Role of cGMP/PKG pathway. *Eur. J. Pharmacol.* **2008**, *587*, 257–266. [CrossRef]
81. Wu, J.; Nakashima, S.; Shigyo, M.; Yamasaki, M.; Ikuno, S.; Morikawa, A.; Takegami, S.; Nakamura, S.; Konishi, A.; Kitade, T.; et al. Antihypertensive constituents in Sanoshashinto. *J. Nat. Med.* **2020**, *74*, 421–433. [CrossRef] [PubMed]
82. Khan, S.A.; Mathews, W.R.; Meisheri, K.D. Role of calcium-activated K+ channels in vasodilation induced by nitroglycerine, acetylcholine and nitric oxide. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 1327–1335. [PubMed]
83. Langton, P.D.; Nelson, M.T.; Huang, Y.; Standen, N.B. Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. *Am. J. Physiol.* **1991**, *260*, H927–H934. [CrossRef] [PubMed]
84. Modzelewska, B.; Drygalski, K.; Hady, H.R.; Kiełczewska, A.; Chomentowski, A.; Koryciński, K.; Głuszyńska, P.; Kleszczewski, T. Resveratrol Relaxes Human Gastric Smooth Muscles Through High Conductance Calcium-Activated Potassium Channel in a Nitric Oxide-independent Manner. *Front. Pharmacol.* **2022**, *13*, 823887. [CrossRef] [PubMed]
85. Giangiacomo, K.M.; Sugg, E.E.; Garcia-Calvo, M.; Leonard, R.J.; McManus, O.B.; Kaczorowski, G.J.; Garcia, M.L. Synthetic charybdotoxin-iberiotoxin chimeric peptides define toxin binding sites on calcium-activated and voltage-dependent potassium channels. *Biochemistry* **1993**, *32*, 2363–2370. [CrossRef]
86. Favaloro, J.L.; Kemp-Harper, B.K. The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc. Res.* **2007**, *73*, 587–596. [CrossRef] [PubMed]
87. Münzel, T.; Holtz, J.; Mülsch, A.; Stewart, D.J.; Bassenge, E. Nitrate tolerance in epicardial arteries or in the venous system is not reversed by N-acetylcysteine in vivo, but tolerance-independent interactions exist. *Circulation* **1989**, *79*, 188–197. [CrossRef]
88. Tsikas, D.; Surdacki, A. Biotransformation of organic nitrates by glutathione S-transferases and other enzymes: An appraisal of the pioneering work by William B. Jakoby. *Anal. Biochem.* **2022**, *644*, 113993. [CrossRef] [PubMed]
89. Abu-Alghayth, M.; Vanhatalo, A.; Wylie, L.J.; McDonagh, S.T.; Thompson, C.; Kadach, S.; Kerr, P.; Smallwood, M.J.; Jones, A.M.; Winyard, P.G. S-nitrosothiols, and other products of nitrate metabolism, are increased in multiple human blood compartments following ingestion of beetroot juice. *Redox Biol.* **2021**, *43*, 101974. [CrossRef]
90. Birnboim, H.C.; Privora, H. Depletion of intracellular glutathione reduces mutations by nitric oxide-donating drugs. *Nitric Oxide* **2000**, *4*, 496–504. [CrossRef]
91. Bergamini, S.; Rota, C.; Canali, R.; Staffieri, M.; Daneri, F.; Bini, A.; Giovannini, F.; Tomasi, A.; Iannone, A. N-acetylcysteine inhibits in vivo nitric oxide production by inducible nitric oxide synthase. *Nitric Oxide* **2001**, *5*, 349–360. [CrossRef] [PubMed]
92. Münzel, T.; Daiber, A.; Mülsch, A. Explaining the phenomenon of nitrate tolerance. *Circ. Res.* **2005**, *97*, 618–628. [CrossRef] [PubMed]
93. Daiber, A.; Oelze, M.; Wenzel, P.; Wickramanayake, J.M.; Schuhmacher, S.; Jansen, T.; Lackner, K.J.; Torzewski, M.; Münzel, T. Nitrate tolerance as a model of vascular dysfunction: Roles for mitochondrial aldehyde dehydrogenase and mitochondrial oxidative stress. *Pharmacol. Rep.* **2009**, *61*, 33–48. [CrossRef] [PubMed]
94. Koenig, A.; Lange, K.; Konter, J.; Daiber, A.; Stalleicken, D.; Glusa, E.; Lehmann, J. Potency and in vitro tolerance of organic nitrates: Partially denitrated metabolites contribute to the tolerance-devoid activity of pentaerythrityl tetranitrate. *J. Cardiovasc. Pharmacol.* **2007**, *50*, 68–74. [CrossRef] [PubMed]
95. Kukovetz, W.R.; Holzmann, S. Mechanisms of nitrate-induced vasodilatation and tolerance. *Eur. J. Clin. Pharmacol.* **1990**, *38* (Suppl. S1), S9–S14. [CrossRef]
96. Müllenheim, J.; Müller, S.; Laber, U.; Thämer, V.; Meyer, W.; Bassenge, E.; Fink, B.; Kojda, G. The effect of high-dose pentaerythritol tetranitrate on the development of nitrate tolerance in rabbits. *Naunyn Schmiedebergs Arch. Pharmacol.* **2001**, *364*, 269–275. [CrossRef]
97. Rutherford, E.; Struthers, A.D. Pentaerythrityl tetranitrate (PETN): A better nitrate? *Eur. Heart J.* **2019**, *40*, e23–e25. [CrossRef]