Vascular Pharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Sodium nitrite causes relaxation of the isolated rat aorta: By stimulating both endothelial NO synthase and activating soluble guanylyl cyclase in vascular smooth muscle

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ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form 12 May 2015
Accepted 29 May 2015
Available online xxxx

Keywords:
Hypertension
Nitrite
Endothelial nitric oxide synthase
Soluble guanylyl cyclase
Nitric oxide

ABSTRACT

Ingestion of dietary nitrites lowers arterial blood pressure in experimental animals and in humans. However, the exact mechanism underlying the hypotensive effect of nitrite remains unclear. The present study compared nitrite-induced responses in rings (with or without endothelium) of aortae of 18–20 weeks old Wistar–Kyoto Rats (WKY) and spontaneously hypertensive (SHR) rats and investigated the underlying mechanism. Relaxations of aortae from WKY and SHR to increasing concentrations (1 nM–100 μM) of sodium nitrite ($NaNO_2$) were determined during sustained contractions to phenylephrine, in the absence and presence of pharmacological agents. The nitrite-induced relaxations were concentration-dependent and larger in SHR than in WKY aortic rings. Inhibition of endothelial nitric oxide synthase (eNOS) and the absence of endothelium decreased nitrite-induced relaxations in both WKY and SHR aortae, indicating the role of endothelium-derived nitric oxide (NO) in the response. The involvement of eNOS was further confirmed by increases in phosphorylation of eNOS at ser1177 in HUVEC cells following treatment with sodium nitrite. The presence of NO scavengers decreased the relaxation to nitrite in both WKY and SHR preparations while inhibition of soluble guanylyl cyclase (sGC) abolished the response, indicating that besides producing NO, nitrite also induces relaxation by directly activating the enzyme. Thus, the present study demonstrates that the sensitivity to exogenous nitrite is increased in the aorta of the SHR compared to that of the WKY. The endothelium-dependent component of the relaxation to nitrite involves activation of eNOS with production of endothelium-derived NO, while the endothelium-independent component is due to stimulation of sGC.

1. Introduction

Hypertension is a multi-factorial cardiovascular disease, with a predicted prevalence of 30% worldwide by 2025 [1]. Endothelial dysfunction accompanies the progression of hypertension [2]. It is characterized by the reduced bioavailability of nitric oxide (NO), an endothelium-derived mediator which participates majorly to local vasomotor control [3,4].

In the blood vessel wall, NO is formed mainly in the endothelium by endothelial NO synthase (eNOS, isoform: NOS III) [5–8]. It acts as a paracrine regulator of the tone of the underlying vascular smooth muscle tone where it activates soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), which in turn inhibits the contractile process, thus causing vasodilatation [4]. The bioavailability of endothelium-derived NO is limited by the scavenging action of superoxide anions leading to formation of peroxynitrite [9,10] or by rapid oxidation of NO by hemoglobin found in the blood and tissues into more stable metabolites such as nitrate (NO_3^-) and nitrite (NO_2^-) [11]. In mammals in particular, nitrite is formed through the oxidation of NOS-derived nitric oxide [12]. In addition, nitrite in the body also originates from processed food (in which it is used as preservative [13]) but from vegetables [14], as well as from the reduction of nitrate by commensal bacteria in the digestive system [13].

The relaxing effect of sodium nitrite has been demonstrated by Furchgott and Bhadrakam in epinephrine-contracted rabbit aortic rings [15]. Later, he and his co-workers showed that sodium nitrite induced an endothelium-independent photorelaxation when Krebs' solution containing sodium nitrite was irradiated with UV light. The studies led to the conclusion that irradiation potentiated NO production from sodium nitrite, resulting in relaxation of the vascular smooth muscle

Abbreviations: cGMP, cyclic guanosine monophosphate; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; L-NAME, N_ω-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; WKY, Wistar–Kyoto rat.

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http://dx.doi.org/10.1016/j.vph.2015.05.014
1537-1891/

cells. This nitrite-dependent photorelaxation was enhanced by scavenging of superoxide anions by superoxide dismutase and was reduced by the generation of superoxide anions and hemoglobin, supporting the existence of a conversion pathway from NO_2^- to NO [16,17]. The conversion from NO_2^- to NO was further confirmed with electron paramagnetic resonance (EPR) spectroscopy measuring the hemoglobin(Hb)–NO as an index of circulating NO in the whole blood [18]. Orally administered nitrite produced an apparent EPR signal 5 min after treatment [18]. However, no increase in Hb–NO was observed after intravenous injection of sodium nitrite [19]. This difference in Hb–NO formation between oral and intravenous treatment may be due to the acid decomposition pathway of nitrite in the stomach, as well as to NO scavenging by hemoglobin in the erythrocytes [20].

Although nitrite can be recycled to bioactive NO by several pathways [deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, cytochrome P_{450} enzymes, mitochondrial respiratory chain enzymes, aldehyde oxidase, carbonic anhydrase, acidic disproportionation and reducing agents (ascorbate and polyphenols)] during ischemic or hypoxic events [21], such reduction requires conditions of low oxygen concentration and pH not readily achieved in the body. Hence, the contribution of endogenous nitrite to physiological vasodilatations remains questionable. However, supplementation with exogenous nitrite exerts an antihypertensive effect under normal physiological conditions. For example, diastolic arterial blood pressure of healthy volunteers was lowered by sodium nitrite supplementation [22]. Likewise, nitrite is antihypertensive in the rat [23]. Thus, the use of exogenous nitrite is a potential treatment for hypertension associated with NO deficiency.

Therefore, the present study aimed to elucidate the mechanism underlying the vasodilator effect of sodium nitrite in aortae of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats.

2. Materials and methods

2.1. Animals

The experimental procedures were approved by the Animal Care and Ethics Committee of the University of Malaya. Eighteen to twenty weeks old male WKY and SHR rats were purchased from BioLASCO (Taipei, Taiwan). The animals were housed in individually ventilated cages at ambient temperature. All animals were supplied with normal rat chow and tap water ad libitum.

2.2. Arterial blood pressure measurement

The arterial systolic blood pressure of the rats was measured by the tail-cuff method (NIBP Monitoring System, IITC Inc., Woodland Hills, CA, USA) before sacrifice. The animals were restrained in a pre-warmed chamber (28–30 °C) for at least 30 min before the blood pressure measurement was carried out. At least five to six successive measurements were recorded and the average values of these readings were calculated and reported.

2.3. Tissue preparation

The rats were sacrificed by carbon dioxide (CO_2) inhalation and their descending thoracic aortae were harvested. Adventitial fat and connective tissue were removed before the aorta was cut into rings (3–4 mm length). The rings were then suspended in organ chambers containing modified Krebs physiological salt solution (control solution in mM: NaCl 118.93, $NaHCO_3$ 25.00, $MgSO_4$ 1.18, KCl 4.69, KH_2PO_4 1.03, Glucose 11.10, $CaCl_2$ 2.38). The solution was continuously aerated with a 95% O_2–5% CO_2 gas mixture and maintained at 37 °C. In some of the rings, the endothelium was removed by gentle rolling using the tip of a forceps inserted into the lumen [24].

The rings were then connected to a force transducer (Grass Instrument Co, Quincy, MA, USA) and their isometric tension was recorded [Mac Lab recording system (AD Instruments, Sydney, Australia)]. The preparations were allowed to equilibrate for 60 min at a resting tension of 1.0 g. The viability of the rings was tested thereafter by the addition of 80 mM KCl solution repeatedly until reproducible contractions were achieved. The endothelial integrity was verified by the addition of 100 nM phenylephrine into the organ chamber, followed by 10 μM acetylcholine before the actual experiments. Rings exhibiting more than 60% relaxation during phenylephrine-induced contractions were considered to contain sufficient endothelium while relaxations less than 5% indicated successful removal of the endothelium.

2.4. Pharmacological studies

Relaxation was obtained to cumulative concentrations (1 nM–100 μM) of sodium nitrite ($NaNO_2$). The role of eNOS in modulating responses to sodium nitrite was determined by the removal of the endothelium or incubation with the NOS inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM [25]). To determine the contribution of NO-formation to nitrite-induced relaxations, the rings were pre-incubated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO, 100 μM [26]), a NO scavenger. To assess the contribution of guanyl cyclase, the rings were pre-incubated with the inhibitor of the enzyme 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM [25]). To investigate if the smooth muscle sensitivity of the arteries to NO differed between strains, the relaxation of the vascular rings was tested with the exogenous NO donor, sodium nitroprusside (SNP, 1 nM–100 μM). The pharmacological inhibitors were introduced into the organ chamber 30 min before obtaining concentration–relaxation curves to $NaNO_2$; at the concentration used, they did not affect the resting tension of the aortic tissues (data not shown).

2.5. Measurement of cGMP level

The total cGMP in aortic tissue was determined using commercially available assay kits (Direct cGMP ELISA Kit; Enzo Life Sciences Inc., Farmingdale, NY, USA). The assay was conducted according to the manufacturer's instructions. Briefly, aortae from WKY and SHR were pre-incubated with sodium nitrite (10 μM) in the absence and presence of inhibitors prior to the assay. The tissues were then homogenized in 0.1 M hydrochloric acid and the supernatant was used to perform the assay.

2.6. Determination of phosphorylated eNOS expression

Confluent human umbilical vein endothelial cells (HUVECs) seeded on six-well plates were exposed to serum-free culture medium for 4 h before any treatment was initiated. The cells were treated with calcium ionophore A23187 (1 μM) and sodium nitrite (10 μM) for 30 min. To investigate the involvement of eNOS in the response to sodium nitrite, the cells are pre-treated with L-NAME (100 μM) 30 min prior to addition of sodium nitrite. At the end of the treatments, the cells were harvested in cold RIPA buffer (1 μg/ml leupeptin, 5 μg/ml aprotonin, 100 μg/ml PMSF, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM EDTA, 1 mM NAF, 2 mg/ml glycerol phosphate) and the cell lysates were centrifuged at 15,000 *g* for 30 min. The supernatant was then collected for Western blotting. Modified Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA) was applied to determine the protein concentration of the supernatant. Protein samples (15 μg) for each lane were separated on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 110 V for 2 h. The blots were then blocked for non-specific protein binding with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBS) at room temperature with gentle shaking. After that, the blots were incubated with

Please cite this article as: W.C. Ling, et al., Sodium nitrite causes relaxation of the isolated rat aorta: By stimulating both endothelial NO synthase and activating soluble guanylyl cyclase in vascular smooth muscle, Vascul. Pharmacol. (2015), http://dx.doi.org/10.1016/j.vph.2015.05.014