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Nitrite- and nitroxyl-induced relaxation in porcine coronary (micro-) arteries: Underlying mechanisms and role as endothelium-derived hyperpolarizing factor(s)

Ilse P.G. Botden, Wendy W. Batenburg, René de Vries, Janneke G. Langendonk, Eric J.G. Sijbrands, A.H. Jan Danser*

Division of Pharmacology and Vascular Medicine, Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

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ABSTRACT

To investigate the vasorelaxant efficacy of nitrite and nitroxyl (HNO) in porcine coronary (micro)arteries (PC(M)As), evaluating their role as endothelium-derived hyperpolarizing factors (EDHFs), preconstricted PCAs and PCMAs were exposed to UV light (a well-known inductor of nitrite; wave-length: 350–370 nm), nitrite, the HNO donor Angeli's salt, or bradykinin. UV light-induced relaxation of PCAs increased identically after endothelium removal and endothelial nitric oxide (NO) synthase (eNOS) blockade. UV light-induced relaxation diminished during Na^+-K^+-ATPase inhibition and *S*-nitrosothiol-depletion, and disappeared during NO scavenging with hydroxocobalamin or soluble guanylyl cyclase (sGC) inhibition with ODQ. Nitrite-induced relaxation of PCAs required millimolar levels, i.e., >1000 times endogenous vascular nitrite. Angeli's salt relaxed PCMAs more potently than PCAs, and this was due to the fact that HNO directly activated sGC in PCMAs, whereas in PCAs this occurred following its conversion to NO only. sGC activation by NO/HNO resulted in Na^+-K^+-ATPase stimulation and K_v channel activation. The HNO scavenger L-cysteine blocked bradykinin-induced relaxation in PCAs, and potentiated it in PCMAs. The latter did not occur in the presence of hydroxocobalamin, suggesting that it depended on L-cysteine-induced generation of vasorelaxant *S*-nitrosothiols. In all experimental setups, incubation with red wine extract mimicked the effects of ODQ. In conclusion, nitrite, via its conversion to NO and *S*-nitrosothiols, and HNO, either directly, or via its conversion to NO, mediate relaxant effects involving the sGC-cGMP pathway, Na^+-K^+-ATPase and/or K_v channels. Red wine extract counteracts these beneficial effects. NO blocks nitrite activation, and HNO, but not nitrite, may act as EDHF in the coronary vascular bed.

1. Introduction

Vascular nitric oxide (NO) originates from de novo synthesis by endothelial NO synthase (eNOS) and/or storage forms of NO. The latter comprise nitrite, nitrate, *S*-nitroso compounds, and *N*-nitroso compounds [1]. Their presence is not limited to endothelial cells [2]. *S*-Nitrosothiols activate endothelial intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels (IK_{Ca}, SK_{Ca}), and via soluble guanylyl cyclase (sGC), smooth muscle Na^+-K^+-ATPase [2–4]. Bradykinin dilates porcine coronary arteries (PCAs), at least in part, by stimulating the release of *S*-nitrosothiols from endothelial cells [2–4], and *S*-nitrosothiols may thus act as endothelium-derived hyperpolarizing factors (EDHF). Importantly, *S*-nitrosothiols, by reacting with other thiols at physiological pH, yield nitroxyl (HNO), a recently discovered EDHF [5–7]. In addition, HNO can be produced by NOS in the absence of tetrahydrobiopterin [8].

Light-induced vasorelaxation ('photorelaxation') also depends on *S*-nitrosothiols, which decompose to a disulfide and NO [1,2]. Additionally, nitrite may undergo photolysis to NO [9,10]. However, its photoactivity is about two orders of magnitude lower than that of *S*-nitrosothiols [1], and apparent only when exposing vessels to UV light. Two different sources contributing to NO release following light exposure (i.e., *S*-nitrosothiols and nitrite) may explain why

Abbreviations: 4-AP, 4-aminopyridine; CRC, concentration–response curve; DEA-NONOate, diethylamine NONOate; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NO synthase; IK_{Ca}/SK_{Ca}, intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels; HNO, nitroxyl; K_v, voltage-dependent K^+ channel; L-NAME, N^{ω}-nitro-L-arginine methyl ester HCl; L-NMMA, N^{ω}-methyl-L-arginine acetate salt; NO, nitric oxide; NO_2, nitrite; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one; PCA, porcine coronary artery; PCMA, porcine coronary microartery; PHMBA, *p*-hydroxymercurobenzoic acid; RWE, red wine extract; sGC, solubel guanylyl cyclase; TRAM34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole.

* Corresponding author at: Division of Pharmacology and Vascular Medicine, Department of Internal Medicine, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. Tel.: +31 10 7043540; fax: +31 10 7044733.
E-mail address: a.danser@erasmusmc.nl (A.H.J. Danser).

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visible light and UV light induce relaxant responses that differ in length and intensity [11]. The *S*-nitrosothiol-dependent response is transient and can be demonstrated a second time only when the vessel is allowed to recover in the dark ('repriming') [2,11]. Repriming for the nitrite-dependent response has not yet been investigated in detail. In vivo, nitrite is believed to be the largest vascular storage pool of NO. Its reduction to NO depends on the reductase activity of deoxyhemoglobin, which is increased under hypoxic conditions [12]. Interestingly, *S*-nitrosothiols have also been described to be generated from nitrite-released NO [13,14].

Red wine consumption is associated with a reduced risk of cardiovascular disease [15,16], possibly because the polyphenols in red wine induce relaxation via NO and/or EDHF [17–19]. Yet, acutely red wine extract (RWE) inactivates sGC, most likely as a consequence of massive NO exposure [18].

It was the aim of the present study to investigate the mechanisms underlying nitrite- and HNO-induced vasorelaxation in coronary arteries, evaluating their potential role as EDHFs. PCAs were exposed to UV light in the presence or absence of suppressors of *S*-nitrosothiols, NO and EDHF. This allowed us to delineate the vasodilator actions of nitrite, since nitrite is the mediator of UV light-induced vasodilation [11]. Second, the responses to the HNO donor Angeli's salt [5,6] were evaluated in PCAs and porcine coronary microarteries (PCMAs). Third, we verified whether nitrite and/or HNO act as endogenous EDHF by studying the responses to bradykinin in PCAs and PCMAs, two vessel types where, respectively, NO and EDHF are the predominant mediators of the bradykinin-induced relaxant response [20,21]. Finally, among the many inhibitors of EDHF pathways, we tested the consequences of sGC inactivation by RWE.

2. Methods

2.1. Drugs

N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME), N^{ω}-methyl-L-arginine acetate salt (L-NMMA), *p*-hydroxymercurobenzoic acid (PHMBA), ethacrynic acid, L-cysteine, indomethacin, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1one (ODQ), hydroxocobalamin, 4-aminopyridine, ouabain, glibenclamide, iberiotoxin, apamin, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM34), bradykinin, diethylamine NONOate (DEA-NONOate) and substance P were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Angeli's salt was obtained from Calbiochem (Darmstadt, Germany). RWE (Provinols, Seppic, France) was a kind gift of Unilever, the Netherlands. This RWE contained 632 mg polyphenols/g, determined as gallic acid equivalents using Folin Ciocalteu reagent [22]. Stock solutions of ODQ, ouabain, TRAM34, indomethacin, ethacrynic acid and glibenclamide were made in DMSO. Stock solutions of L-cysteine (300 mmol/l), Angeli's salt (10 mmol/l; dissolved in 0.01 mol/l NaOH) and DEA/NONOate (10 mmol/l) were prepared fresh daily. All subsequent dilutions and other drugs were made in distilled water. Solutions containing Angeli's salt and DEA-NONOate were stored in the dark.

2.2. Tissue collection

Pig hearts ($n = 105$) were collected at the slaughterhouse and brought to the laboratory in cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. The right proximal (internal diameter 2–3 mm; PCAs) and distal (internal diameter 500–600 μm; PCMAs) coronary arteries were obtained from the hearts and stored in cold, oxygenated Krebs bicarbonate solution for 12–36 h. PCAs were then cut into segments of ≈4 mm length suspended on stainless steel hooks in 15 ml-organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2 and maintained at 37 °C. To remove the endothelium in some segments, the ring was gently rolled back and forward over physiological saline-loaded filter paper after the tips of a pair of watchmaker forceps had been inserted into the lumen. Endothelium-removal was confirmed by the disappearance of the relaxation to bradykinin after U46619-preconstriction. PCMAs were cut into segments of ≈2 mm length and mounted in a Mulvany myograph with separated 6-ml organ baths, aerated with 95% O_2/5% CO_2 and maintained at 37 °C.

2.3. Organ bath studies

Nitrite-induced vasorelaxation was investigated by exposing PCAs to UV-light, a well-known nitrite inductor. A comparison was made versus polychromatic light. These experiments were performed in the dark. To study HNO-induced vasorelaxation, as well as its potential role as EDHF, vessels were exposed to Angeli's salt, an HNO donor, or bradykinin. All experiments were repeated in presence of inhibitors of EDHF pathways, including the HNO scavenger L-cysteine and the sGC inactivator RWE.

Vessel segments were allowed to equilibrate for at least 30 min, and the organ bath fluid was refreshed every 15 min during this period. In PCAs, changes in contractile force were recorded with a Harvard isometric transducer (South Natick, MA, USA). The PCA vessel segments were stretched to a stable force of 15 mN. The tension of the PCMA vessel segments was measured by Powerlab with Labchart Software and was normalized to 90% of the estimated diameter at 100 mmHg effective transmural pressure [23]. The vessel segments were exposed to 30 mmol/l KCl twice and, subsequently, to 100 mmol/l KCl to determine the maximal contractile response. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the NOS inhibitors L-NAME (100 μmol/l [3]) and L-NMMA (100 μmol/l [24]), the *S*-nitrosothiol-depleting agents PHMBA (10 μmol/l [2]) and ethacrynic acid (50 μmol/l [2]), L-cysteine (3 mmol/l [7]), the cyclooxygenase (COX) inhibitor indomethacin (10 μmol/l [4]), the sGC inhibitor ODQ (10 μmol/l [7]), the NO scavenger hydroxocobalamin (200 μmol/l [4]), RWE (30 μg/ml [18]), K^+ (20 mmol/l KCl [3]), the voltage-gated K^+ (K_v) channel inhibitor 4-aminopyridine (5 mmol/l in the photorelaxation experiments [2], 0.5 mmol/l in all other experiments due to an instable preconstriction curve at a higher concentration), the Na^+-K^+-ATPase inhibitor ouabain (0.5 mmol/l [25]), the ATP-sensitive K^+-channel (K_{ATP}) inhibitor glibenclamide (1 μmol/l [26]), the BK_{Ca} inhibitor iberiotoxin (100 nmol/l [27]), the SK_{Ca} inhibitor apamin (100 nmol/l [28]) or the IK_{Ca} inhibitor TRAM34 (10 μmol/l [2]). After 30 min of incubation with RWE, the organ bath fluid was refreshed.

For the light experiments, PCAs were preconstricted with the thromboxane A_2 analogue U46619 (1 μmol/l) and exposed six times for 5 min to light (wavelength: 350–370 nm) from a UV source (Omnilux UV-Röhre 18 W, G13, 600 mm × 26 mm, T8) or from a halogen dissection lamp omitting polychromatic light. Each exposure was followed by a period in the dark of 2–30 min. After six light exposures, the vessels were subjected to 100 nmol/l bradykinin. In case of sGC inhibition, ODQ was added to the organ bath fluid just before each UV light exposure, since preliminary experiments showed degradation of ODQ due to UV light exposure.

For the experiments not involving photorelaxation, vessels were preconstricted with U46619 (1 μmol/l for PCAs, 0.01–1 μmol/l for PCMAs) to ~70–80% of the maximum constriction, and concentration–response curves (CRCs) were constructed to bradykinin (0.1 nmol/l to 1 μmol/l), Angeli's salt (sodium tiroxodinitrate; 0.1 nmol/l to 10 μmol/l), the NO donor