European Journal of Pharmacology 814 (2017) 294–301

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

Angeli's Salt, a nitroxyl anion donor, reverses endothelin-1 mediated vascular dysfunction in murine aorta

Brandi M. Wynne[a,b,*,1], Hicham Labazi[a,c,**,1,2], Zidonia N. Carneiro[a,3], Rita C. Tostes[d], R. Clinton Webb[a,4]

[a] Department of Physiology, Medical College of Georgia at Augusta University, Augusta, GA 30912, United States
[b] Department of Medicine, Renal Division, Emory University, 615 Michael St. Ste 605C, Atlanta, GA 30322, United States
[c] Center for Cardiovascular Research, The Research Institute at Nationwide Children's Hospital, Columbus, OH 43215, United States
[d] Pharmacology Department, Medical School of Ribeirão Preto, University of São Paulo, Av Bandeirantes 3900, Ribeirão Preto, SP 14049-900, Brazil

ARTICLE INFO

Keywords:
HNO
Angeli's Salt
Nitroglycerin
Endothelin-1
Vascular dysfunction

ABSTRACT

Nitroglycerin (Gtn) is a treatment for cardiovascular patients due to its vasodilatory actions, but induces tolerance when given chronically. A proposed mechanism is the superoxide (O_2^-)-oxidative stress hypothesis, which suggests that Gtn increases O_2^- production. Nitric oxide (NO) exists in three different redox states; the protonated, reduced state, nitroxyl anion (HNO) is an emerging candidate in vascular regulation. HNO is resistant to scavenging and of particular interest in conditions where high levels of reactive oxygen species (ROS) exist. We hypothesize that treatment with Gtn will exacerbate endothelin 1 (ET-1) induced vascular dysfunction *via* an increase in ROS, while treatment with Angeli's Salt (AS), an HNO donor, will not. Aorta from mice were isolated and divided into four groups: vehicle, ET-1 [0.1 μM, 1 μM], ET-1 + Gtn [Gtn 1 μM] and ET-1 + AS [AS 1 μM]. Concentration response curves (CRCs) to acetylcholine (ACh) and phenylephrine (Phe) were performed. Aorta incubated with ET-1 (for 20–22 h) exhibited a decreased relaxation response to ACh and an increase in Phe-mediated contraction. Aorta incubated with AS exhibited a reversal in ET-1 induced vascular and endothelial dysfunction. ET-1 increased ROS in aortic vascular smooth muscle cells (VSMCs), visualized by dihydroethidium (DHE) staining. AS incubated reduced this ROS generation, yet maintained with Gtn treatment. These data suggest that aorta incubated with the HNO donor, AS, can reverse ET-1 mediated vascular dysfunction, which may be through a decrease or prevention of ROS generation. We propose that HNO may be vasoprotective and that HNO donors studied as a therapeutic option where other organic nitrates are contraindicative.

1. Introduction

Nitroglycerin (glyceryl nitrate, Gtn), was proposed by Murrell in 1879 as a treatment for angina (Murrell, 1879; Frame et al., 2002). Gtn continues to be used for various conditions, including angina, heart failure and other acute cardiovascular crises. While this and other organic nitrovasodilators improve cardiac output by decreasing preload and afterload on the heart, and inducing vasodilation, these compounds are also of limited clinical efficacy. They induce tolerance when given over time, which has been demonstrated clinically and in various animal models (Munzel et al., 2014). Additionally, when conditions of increased reactive oxygen species (ROS) are present, they also have a diminished vasoprotective effect, and even induce vascular wall damage.

There has been considerable research regarding the one electron reduced congener of nitric oxide (NO), nitroxyl anion (NO^-) (Irvine et al., 2003, 2007, 2008; Favaloro and Kemp-Harper, 2007; Andrews et al., 2009; Chin et al., 2016; Miao and King, 2016; Shoman and Aly, 2016). It was previously thought that NO^- exists as an anion (pKa 4.7) at a physiological pH; however, this assumption was corrected in 2002,

* Correspondence to: Emory University, 615 Michael St. Suite 605C, Whitehead Research Building, Emory University, Atlanta, GA 30322, United States.
** Co-first author.
E-mail addresses: bwynne@emory.edu (B.M. Wynne), Hicham.Labazi@nationwidechildrens.org (H. Labazi), znunes@hotmail.com, znunes@hotmail.com (Z.N. Carneiro), rtostes@usp.br (R.C. Tostes), CWEBB@augusta.edu (R.C. Webb).
[1] These authors contributed equally to this manuscript.
[2] Current address: Center for Cardiovascular and Pulmonary Research, The Research Institute at Nationwide Children's Hospital, 700 Children's Dr., Columbus, OH 43205, United States.
[3] Department of Physiology, Augusta University, 1120 15th Street, CA3099, Augusta, GA 30912, United States.
[4] Department of Physiology, Augusta University, 1120 15th Street, CA3123, Augusta, GA 30912, United States.

http://dx.doi.org/10.1016/j.ejphar.2017.08.024
Received 28 November 2016; Received in revised form 15 August 2017; Accepted 18 August 2017
Available online 20 August 2017
0014-2999/

when investigators determined the actual pKa to be around 11.4. At physiological pH, NO^- exists as HNO (Gratzel, 1970; Bartberger, 2001; Shafirovich and Lymar, 2002; Fukuto et al., 2005a). It was also determined that this conjugated weak acid, HNO, can cross cellular membranes, leading investigators to divert research to this understudied molecule (Bartberger, 2001). Although the comparative mechanisms between HNO and NO are still being investigated, it is widely accepted that their physiology, pharmacology and biochemistry are vastly different (Fukuto et al., 2005a, 2005b; Andrews et al., 2009; Favaloro and Kemp-Harper, 2009; Wynne et al., 2012).

This phenomenon can be seen when using the HNO/NO^- donor, Angeli's Salt (AS). Various studies have used AS to study HNO-mediated vasorelaxation. One of the most attractive effects of AS is the lack of tolerance induced (Irvine et al., 2007, 2008, 2010). In functional studies performed using rat aorta, Irvine and colleagues demonstrated that the use of the HNO-donor AS, did not induce tolerance as compared to Gtn (Irvine et al., 2007, 2010). Additionally, they also revealed that AS did not exhibit a cross tolerance to Gtn (Irvine et al., 2010). A recent study has demonstrated a similar phenomenon *in vivo*; chronic infusion of AS did not lead to tolerance or endothelial dysfunction (Irvine et al., 2007, 2010). The exact mechanisms of Gtn-induced tolerance have not been fully elucidated; however, several proposed models have been recommended (Munzel et al., 1995; Szocs et al., 2007; Daiber et al., 2009). These mechanisms may overlap, but are suggested to include: reduced biotransformation of the organic nitrates to NO, neurohormonal activation, desensitization of soluble guanylate cyclase (sGC), increased phosphodiesterase 1A1 activity and increased production of ROS (Frame et al., 2002; Fukatsu et al., 2007; Irvine et al., 2007; Szocs et al., 2007; Daiber et al., 2009; Kosmicki, 2009). In fact, there is a growing body of literature showing that administration of organic nitrates not only induces tolerance, but promotes vascular wall damage (Sage et al., 2000; Bartberger, 2001; Schulz et al., 2002; Munzel et al., 2005; Irvine et al., 2008).

It has also been demonstrated that HNO is less reactive as compared to NO, which may lend HNO a superior stability against reactions with ROS or during conditions of high oxidative stress, such as hypertension (Savoia and Schiffrin, 2006, 2007; Schiffrin, 2007; Irvine et al., 2008; Paolocci and Wink, 2009; Switzer et al., 2009). Common mediators of hypertension-induced ROS generation, promotion of oxidative stress and vascular wall damage are angiotensin II (AngII) and endothelin-1 (ET-1) (Wilcox, 2002, 2010; Palm et al., 2010; Pollock and Pollock, 2011; Nguyen Dinh Cat et al., 2013; Gonzalez et al., 2014; Brito et al., 2015; Montezano et al., 2015). In this manuscript, we sought to determine whether the HNO/NO^- donor (AS) would alleviate ET-1-induced vascular dysfunction. We hypothesized that treatment with Gtn will exacerbate ET-1 vascular dysfunction *via* an increase in ROS, while AS will not.

To our knowledge, this is the first study to compare the effects of Gtn *vs.* HNO and investigate vascular responses *ex vivo*. The importance of these experiments will further demonstrate how the use of organic nitrates during pathophysiological conditions such as hypertension, where there are significantly increased levels of ROS, may not always be beneficial. Additionally, we will also reveal that using donors for HNO, which has proven to be a more stable molecule and is resistant to scavenging from ROS, may be a better therapeutic option.

2. Materials and methods

Male C57bl/6 mice, weighing between 25 and 30 g were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 12-h light dark cycle, housed five per cage and allowed access to chow and water *ad libitum*. Isoflurane (10%) in oxygen was used for surgeries with carbon dioxide (CO_2) for euthanasia. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia at Augusta University Committee on the Use of Animals in Research and Education.

2.1. Primary cell culture

Aorta from mice were carefully excised and cleaned in sterile Dubelco's Modified Eagle's Medium (high glucose, DMEM), supplemented with 1% penicillin/streptomycin and 30% fetal bovine serum (FBS). Per previous protocols and experimental procedures used in this laboratory, the endothelium was removed and a primary vascular smooth muscle cell (VSMC) culture obtained *via* explant technique (Carrillo-Sepulveda et al., 2010). The VSMCs were maintained in DMEM (low glucose), supplemented with 1% penicillin/streptomycin and 10% FBS under normal cell culture conditions (37 °C, 5% CO_2). Cells were grown to confluency, passed using trypsin and used within 4–6 passages.

2.2. Aortic ring incubation

After euthanasia with CO_2, the aorta was rapidly excised and bathed in ice-cold physiological salt solution (PSS) (NaCl 120 mM, KCl 4.7 mM, KH_2PO_4 1.18 mM, $NaHCO_3$ 14.9 mM, dextrose 5.6 mM, $CaCl_2{\cdot}H_2O$, 0.06 mM EDTA). Increased concentrations of EDTA were used in PSS buffer to aid in preventing the extracellular conversion of HNO to NO. Aorta were carefully isolated and incubated in DMEM (low glucose) supplemented with 1% penicillin/streptomycin. Vessels were incubated overnight in incubator per standard cell culture technique and divided into the following groups: vehicle, endothelin-1 (ET-1, 0.1 μM or 1 μM, Pheonix Pharmaceuticals, Burlingame CA), ET-1 plus the HNO donor, Angeli's Salt (ET-1 + AS, 1 μM, Cayman Chemical, Ann Arbor MI) or ET-1 plus Gtn (ET-1 + Gtn, 1 μM, American Regent, Shirley NY). Vessels were incubated overnight in either vehicle or ET-1 for 20–22 h. During the last hour of incubation, vessels were given two treatments of either AS or Gtn (one treatment at one hour prior and second 30 min prior to functional experiment).

2.3. Functional studies

Aortas were mounted as ring preparations on two stainless steel pins in a myograph (Danish MyoTech, Aarhus, Denmark). Vessels were maintained at 37 °C and continuously aerated with 95% O_2, 5% CO_2 and allowed to stabilize for at least 45 mins, at an optimal passive force of 5.0 mN. After stabilization, tissues were contracted with KCl (120 mM) solution to determine the reactivity of the vascular smooth muscle cells. To determine endothelium viability, contraction was stimulated *via* phenylephrine (Phe; 1 μM) followed by relaxation with acetylcholine (ACh; 10 μM). Vessels were then washed before performing concentration response curves (CRC) and after each CRC. CRCs to ACh in Phe (10 μM)-contracted vessels were performed in the presence of vehicle or the following: tempol (O_2^- scavenger), Iron (III) ^{5,10,15,20}-Tetrakis (4-sulfonatophenyl) porphyrinato, chloride (Fe-TPPS, $ONOO^-$ degradation catalyst). All other chemicals and drugs were purchased from Sigma Aldrich, St. Louis, MO. Force measurements were collected using Chart™ Software (ADI Instruments, Colorado Springs, CO) for PowerLab data acquisition systems (ADI Instruments).

2.4. Dihydroethidium staining

VSMC primary cells were used in determining ROS and O_2^- generation with dihydroethidium (DHE, Invitrogen, California), which oxidizes in the presence of ROS, generating fluorescence (Bruder-Nascimento et al., 2014; Silva et al., 2015). VSMCs were grown to confluence, and then seeded to 6-well plates. Once VSMCs reached 80% confluency, experiments were performed using: vehicle, ET-1 (0.1 μM), ET-1 plus AS (10 μM) and ET-1 plus Gtn (10 μM). VSMCs were treated with ET-1 for 2 min, in the presence of vehicle, AS or Gtn administered simultaneously. After washing 3 times in phosphate buffered saline