Nitric Oxide 45 (2015) 54–64

Contents lists available at ScienceDirect

Nitric Oxide

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

Validation of a method to directly and specifically measure nitrite in biological matrices

Luis E.F. Almeida [a], Sayuri Kamimura [a], Nicholas Kenyon [a,1], Alfia Khaibullina [a], Li Wang [a], Celia M. de Souza Batista [b], Zenaide M.N. Quezado [a,*]

[a] The Sheikh Zayed Institute for Pediatric Surgical Innovation, Division of Pain Medicine, Children's National Medical Center, School of Medicine and Health Sciences George Washington University, Washington, DC 20010, USA
[b] Department of Nutritional Sciences, Howard University, Washington, DC 20059, USA

ARTICLE INFO

Article history:
Received 27 August 2014
Revised 23 October 2014
Available online 28 October 2014

Keywords:
Nitric oxide
Nitrite
Ascorbic acid
Tissue
Plasma
Chloroform

ABSTRACT

The bioactivity of nitric oxide (NO) is influenced by chemical species generated through reactions with proteins, lipids, metals, and its conversion to nitrite and nitrate. A better understanding of the functions played by each of these species could be achieved by developing selective assays able of distinguishing nitrite from other NO species. Nagababu and Rifkind developed a method using acetic and ascorbic acids to measure nitrite-derived NO in plasma. Here, we adapted, optimized, and validated this method to assay nitrite in tissues. The method yielded linear measurements over 1–300 pmol of nitrite and was validated for tissue preserved in a nitrite stabilization solution composed of potassium ferricyanide, N-ethylmaleimide and NP-40. When samples were processed with chloroform, but not with methanol, ethanol, acetic acid or acetonitrile, reliable and reproducible nitrite measurements in up to 20 sample replicates were obtained. The method's accuracy in tissue was ≈90% and in plasma 99.9%. In mice, during basal conditions, brain, heart, lung, liver, spleen and kidney cortex had similar nitrite levels. In addition, nitrite tissue levels were similar regardless of when organs were processed: immediately upon collection, kept in stabilization solution for later analysis or frozen and later processed. After ip nitrite injections, rapidly changing nitrite concentrations in tissue and plasma could be measured and were shown to change in significantly distinct patterns. This validated method could be valuable for investigations of nitrite biology in conditions such as sickle cell disease, cardiovascular disease, and diabetes, where nitrite is thought to play a role.

1. Introduction

A better understanding of nitric oxide (NO) biology has been fraught with a number of challenges. Some of those challenges revolve around the very short life span of NO (100 ms half-life average) [1,2], the complexity of NO-derived decay species in biological tissues and the array of technical difficulties associated with their measurement [3,4]. In blood, NO rapidly reacts with hemoglobin while in tissues it can react with proteins, lipids and metals and generate multiple chemical species such as nitrite, nitrate, peroxynitrite, S-nitrosothiols (RSNOs), N-nitrosamines (RNNOs) and others [5,6]. Recently, it has become clear that several of these NO-derived chemical species can regenerate NO and mediate important physiological functions [6]. For example, physiological roles have been proposed for S-nitrosoalbumin and S-nitrosoglutathione (two major RSNOs) which were demonstrated to mediate NO-dependent vasodilation in humans [7].

Among NO-derived species, nitrite is thought to be an important reserve pool for NO production and a signaling molecule itself [8,9]. Nitrite has been shown to generate NO by several enzymatic mechanisms involving hemoglobin, myoglobin, xanthine oxidase, electron-transport chain complexes and by non-enzymatic reactions with ascorbic acid or polyphenols [10]. In human volunteers, plasma nitrite levels are inversely related to cardiovascular disease risk [11], are decreased after cardiac infarction [12] and nitrite infusions increase arm blood flow [13,14]. In animal models, nitrite supplementation attenuates hypertension induced by nitric oxide synthase blockade [15], protects against pulmonary hypertension

Abbreviations: NO, nitric oxide; RSNOs, S-nitrosothiols; RNNOs, N-nitrosamines; NEM, N-ethylmaleimide; ddH_2O, double-distilled H_2O; KCN, potassium cyanide; NOHb, nitrosyl-hemoglobin; RBC, red blood cells; AS, acidified sulfanilamide; AUC, area under-the-curve; SS, stabilization solution; Alb, albumin; MeOH, methanol; EtOH, ethanol; CF, chloroform; Actn, acetonitrile; NP-40, Nonidet P40 Substitute; ip, intraperitoneal.

* Corresponding author. The Sheikh Zayed Institute for Pediatric Surgical Innovation, Children's National Medical Center, School of Medicine and Health Sciences, George Washington University, 111 Michigan Avenue NW, Washington, DC 20010, USA. Fax: +202 476 1280.
E-mail address: zquezado@childrensnational.org (Z.M.N. Quezado).

[1] In memoriam.

http://dx.doi.org/10.1016/j.niox.2014.10.008
1089-8603/

[16,17] and myocardial infarction [18]. Combined, these data indicate that nitrite is an alternative source of NO production and may play a larger role in conditions such as cardiovascular disease, stroke, or endothelial dysfunction.

Therefore, given the complexity of NO metabolism, a true understanding of NO biology requires highly sensitive and specific assay methods capable of distinguishing among these various NO-derived species in blood and tissues and measuring rapidly changing concentrations. While indirect methods to estimate nitrite levels in biological matrices have been described [19,20], some are coupled with technical challenges and pitfalls [4]. Therefore, the development of assays that directly and specifically measure tissue nitrite levels would greatly advance our understanding of NO/nitrite biology.

Recently, Nagababu and Rifkind [21,22] validated an assay to measure nitrite levels in plasma using a reaction involving acetic and ascorbic acids that specifically reduces nitrite to NO. Coupled with a gas phase chemiluminescence NO detection system, this assay is specific for nitrite over several other S- and N-nitroso species including nitrate, RSNOs, RNNOs, nitrated lipids and nitrated amino acids, thus obviating the need for aliquot co-assays and subtraction [19,20,23]. However, while the acetic/ascorbic acids method is capable of quantifying nitrite in plasma, it has not been validated to measure nitrite levels in biological tissue.

In the present study, we further developed the acetic/ascorbic acids method to directly quantify nitrite in tissue samples. We demonstrated that this method can be used with a nitrite-preserving solution, which reportedly enhances the stability of nitrite in tissue. We also examined the impact of several clarification protocols for tissue processing, and finally documented basal levels and rapidly changing tissue nitrite levels after nitrite administration.

2. Methods

2.1. Animals

The investigational protocol was approved by the Institutional Animal Care and Use Committee of the Children's National Health System. C57BL/6J mice were obtained from Jackson Laboratory (Maine, USA) and housed in 12 h light/dark cycles. Animals were fed standard rodent chow, which contained 3.7 ± 0.1 nmol nitrite/g as measured by the present method (Harlan Teklad LM485, lot 7012042914M) and drank water (from a facility-wide Milli-Q filtration system) containing undetectable nitrite.

2.2. Blood and organ collection

From female mice anesthetized with isoflurane blood was collected via cardiac puncture with a heparin-coated 23G × 3/4 needle fitted into a 1 ml syringe (BD bioscience, Franklin Lakes, NJ). Blood was deposited into a microcentrifuge tube and plasma was separated by centrifugation (500 *g*, 10 min, 4 °C). Plasma was pipetted into a new microcentrifuge tube and re-centrifuged (1000 *g*, 5 min, 4 °C). Plasma was then frozen in dry ice and stored at –80 °C. Organs (brain, heart, lungs, spleen, liver and kidney cortex) were rapidly harvested and placed in 35 mm plastic dishes (BD Bioscience) with ice-cold phosphate buffered saline (Thermo Scientific, Waltham, MA). A 20–40 mg fragment of each organ was weighted and placed in 1 ml of a nitrite-preserving solution [henceforth called stabilization solution [24,25]]. The stabilization solution contained potassium ferricyanide (1.32 g) and N-ethylmaleimide (NEM, 0.0665 g) dissolved in 24.5 ml of double-distilled H_2O (ddH_2O) followed by the addition of 0.5 ml Nonidet™ P40 Substitute (NP40, Sigma-Aldrich, St. Louis, MO) [24,25]. Potassium ferricyanide converts any remaining blood hemoglobin into methemoglobin, which prevents nitrite conversion into nitrate [3,26], blocks formation of nitrosylhemoglobin, which is produced *in vitro* and *in vivo* by NO reaction with deoxyhemoglobin [27] and avoids capture of NO by hemoglobin in the reaction chamber [28]. NEM is a thiol-inactivating agent, which avoids capture of NO by thiols in the reaction chamber [21,22].

2.3. Sample storage

In order to evaluate the impact of sample collection and storage on tissue and plasma nitrite levels, three methods of collection and storage were examined. In the first method, tissue fragments were placed in 1 ml stabilization solution, were processed and nitrite was immediately assayed (labeled *fresh samples*). In the second method, tissue fragments were placed in 1 ml stabilization solution and frozen for processing and assaying ≈1 month later (labeled *SS+frozen*). A third tissue collection strategy was executed by freezing whole organs in dry ice and storing at –80 °C. After 30–45 days, tissue fragments were obtained from these previously frozen tissues, placed in freshly-prepared stabilization solution and processed for nitrite assay (labeled *frozen*). Plasma samples were also processed with stabilization solution which had the same final composition as earlier but was prepared in 4.5 ml of ddH_2O plus 0.5 ml Nonidet™ P40 Substitute (fivefold concentrated). This concentrated nitrite-preserving solution was dissolved 1:4 (v/v) with plasma so that its final concentration was equal to the nitrite-preserving solution used for tissue samples. Importantly, all three collection and storage strategies were executed in the same animal cohort.

2.4. Sample processing

Regardless of collection and storage method, all tissue samples were processed for analysis as follows. Samples were thawed at 4 °C for 1–2 h (if samples had been frozen), multiple 2.3 mm zirconia–silica beads and a single 1/4″ ceramic sphere (MP Biomedicals, Santa Ana, CA) were added, and the sample was placed in a tissue homogenizer (MP Biomedicals) for a 1 min rapid agitation twice. Preliminary results indicated that this bead combination produced the most consistent tissue disruption. After disruption, samples were spun in a refrigerated centrifuge (4 °C) at 15,000 *g* for 15 min. Further sample clarification was investigated by transferring 600 µl of this centrifuged solution into a new microcentrifuge tube and adding methanol, ethanol, acetic acid, acetonitrile (all in 1:1 dilutions with samples) or chloroform (1:10 dilution). Independently of the clarification agent used, all samples were vortexed and incubated for 5–10 min at room temperature. After incubation, samples were centrifuged at 17,000 *g* for 30 min (4 °C) and clear supernatant (obtained with all agents used) was transferred to a new tube and stored at –80 °C for later use. Plasma samples (with stabilization solution) were processed with chloroform only.

2.5. Nitrite assay

Processed samples were thawed at 4 °C, mixed by inversion and centrifuged (15,000 *g*, 10 min, 4 °C). Particularly for samples processed with chloroform, no further protein precipitation was observed after this centrifugation step. Nitrite was measured using the method of Nagababu and Rifkind [21,22] using a Sievers 280i analyzer (GE Analytical Instruments, Boulder, CO) and its acquisition software (Liquid®, version 3.22). Briefly, the reaction chamber attached to the NO analyzer contained 7 ml of glacial acetic acid plus 1 ml of a 480 mM ascorbic acid solution (prepared daily by dissolving ascorbic acid in water; final concentration = 60 mM). Although this nitrite-detection method is resistant to protein-induced foaming [21,22], better results were obtained by adding 200 µl of a 1:30 dilution of antifoam SE-15 (Sigma-Aldrich) to the reaction chamber.