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Review

Measurement of circulating nitrite and *S*-nitrosothiols by reductive chemiluminescence☆

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Abstract

Considerable disparities in the reported levels of basal human nitrite and *S*-nitrosothiols (RSNO) in blood have brought methods of quantifying these nitric oxide (NO) metabolites to the forefront of NO biology. Ozone-based chemiluminescence is commonly used and is a robust method for measuring these species when combined with proper reductive chemistry. The goal of this article is to review existing methodologies for the measurement of nitrite and RSNO by reductive chemiluminescence. Specifically, we discuss in detail the measurement of nitrite and RSNO in biological matrices using tri-iodide and copper(I)/cysteine-based reduction methods coupled to chemiluminescence. The underlying reaction mechanisms, as well as the potential pitfalls of each method are discussed.

Keywords: Reviews; Nitric oxide; *S*-Nitrosohemoglobin; Tri-iodide; Copper(I)/cysteine

Contents

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1. Introduction

Nitric oxide (NO) is a reactive gaseous molecule that plays a critical role in both physiological and pathological vascular signaling. In blood vessels, the diatomic free radical is synthesized by the endothelial isoform of the enzyme nitric oxide synthase (eNOS) using L-arginine as a substrate and NADPH, tetrahydrobiopterin, and oxygen as cofactors [1–3]. Once synthesized in the endothelium, NO can diffuse into underlying smooth muscle cells where it activates soluble guanylyl cyclase, ultimately resulting in the relaxation of the smooth muscle cells and promoting vasodilation. The majority of the NO that does not diffuse abluminally reacts rapidly ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$) with hemoglobin by the following equations (Eqs. (1) and (2)):

$$\text{NO} + \text{oxyhemoglobin}(Fe^{2+}) \rightarrow \text{nitrate}(NO_3^-) + \text{methemoglobin}(Fe^{3+}) \quad (1)$$

$$\text{NO} + \text{deoxyhemoglobin}(Fe^{2+}) \rightarrow \text{iron-nitrosylhemoglobin(Hb-NO)} \quad (2)$$

NO is oxidized to the inert metabolite nitrate by its reaction with oxyhemoglobin, while reaction of NO with deoxyhemoglobin results in the formation of iron-nitrosyl hemoglobin [4]. A fraction of NO produced by eNOS in the vasculature (approximately 20%) escapes inactivation by hemoglobin and is oxidized to nitrite (NO_2^-) in the plasma by a reaction catalyzed by ceruloplasmin [5], modifies proteins and lipids to form low concentrations of *N*-nitrosamines, *S*-nitrosothiols (RSNO), and nitrated lipids. The significance of these lower-yield reaction products is the subject of active investigation, as they may serve as stable storage forms of NO that may later be bioactivated to mediate endocrine NO signaling, distal from the original site of NO production.

A growing number of studies in human subjects and animal models suggest that NO may indeed mediate endocrine signaling. For example, inhaled NO, in addition to mediating pulmonary vasodilation, has been shown to limit myocardial infarction in mice [6], increase urinary blood flow in anesthetized pigs [7], decrease systemic vascular resistance in anesthetized sheep [8], and increase blood flow in the human forearm when eNOS is inhibited [9]. However, the circulating NO species responsible for this endocrine behavior remains unclear. Stamler and co-workers proposed that NO-dependent modification of the β-cysteine-93 of hemoglobin to form *S*-nitrosated hemoglobin is responsible for this activity [10]. We have recently proposed that the NO oxidation product, nitrite, mediates this effect and is a vascular store of NO that is enzymatically reduced to NO by deoxygenated hemoglobin along the physiological oxygen and pH gradient [11,12].

The development of accurate and reproducible methodologies to isolate and quantify NO species is paramount to the ongoing debate surrounding the identity of the endocrine storage form and endocrine NO signaling in vivo. In the last decade, published levels of NO species have been as variable as the different methods used to measure them [13,14], reinforcing the need for extensively validated and robust methodologies. For example, basal concentrations of *S*-nitrosohemoglobin (SNO-Hb) in blood have been reported to range from undetectable [15] to 7 μM [16], and in plasma, reported levels of nitrite have ranged from undetectable [17] to 26 μM [18]. The vast differences in reported levels may be largely attributed to variations in sample processing techniques, the different assays employed, and nitrite contamination during sample preparation. Also inherent to the problem are the debated effects of sample pretreatments used to isolate specific species and the specificity of assays for the range of NO species [19,20].

Quantification difficulties arise when measuring NO species in blood for a variety of reasons. The pervasive nature of nitrite in the laboratory, present in everything from glassware to tap water, poses countless contamination problems leading to overestimation of nitrite concentration. However, the transient nature of nitrite in blood due to its reaction with hemoglobin leads to rapid degradation and underestimated values if not experimentally addressed [14,21,22]. Further, many NO-modified proteins are present at low levels in blood, and their measurement requires the use of highly sensitive methods with corresponding low detection limits. For example, the low basal levels of SNO-Hb and SNO-albumin preclude the use of the traditional Griess–Saville assay for their measurement in plasma and blood as this assay has quantitation limits in the lower μM-range in biological matrices. Thus, the ideal assay would be a method that possesses high sensitivity and specificity coupled with sample treatment that limits the possibility for contamination and degradation in biological samples. Ozone-based chemiluminescence coupled with a variety of reductive methods satisfies these characteristics and will be the focus of the rest of this review.

2. Ozone-based chemiluminescence is a sensitive method for NO measurement

Although many methods and analytical techniques are employed to measure NO and its metabolites, in recent years gas-phase ozone-based chemiluminescence, utilizing machines such as Sievers' nitric oxide analyzer (NOA) (Model 280i) and Eco Physics' CLD 60 Series NO_x analyzers, has emerged as the most commonly used assay for NO detection due to its specificity, sensitivity, versatility and dependable operating performance. The principle of chemiluminescent NO detection is based on the rapid reaction of NO in the gas phase with ozone (O_3), which yields NO_2^* in an excited state. As the excited electron returns to its ground state, a photon is emitted and is detected as chemiluminescence ($h\nu$). (Eqs. (3) and (4)).

$$\text{NO} + O_3 \rightarrow NO_2^* + O_2 \quad (3)$$

$$NO_2^* \rightarrow NO_2 + h\nu \quad (4)$$

This emitted light is detected and amplified by a photomultiplier tube (PMT), to generate an electrical signal. The specificity of this method for NO is due to the unique properties of the NO molecule, including its ability to exist as a gas and its rapid reaction rate with ozone [23].