Free Radical Biology and Medicine 63 (2013) 51–64

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Efficient nitrosation of glutathione by nitric oxide ☆

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ARTICLE INFO

Article history:
Received 17 April 2012
Received in revised form
24 January 2013
Accepted 27 April 2013
Available online 7 May 2013

Keywords:
S-nitrosation
Nitrosoglutathione
Nitric oxide
Free radicals

ABSTRACT

Nitrosothiols are increasingly regarded as important participants in a range of physiological processes, yet little is known about their biological generation. Nitrosothiols can be formed from the corresponding thiols by nitric oxide in a reaction that requires the presence of oxygen and is mediated by reactive intermediates (NO_2 or N_2O_3) formed in the course of NO autoxidation. Because the autoxidation of NO is second order in NO, it is extremely slow at submicromolar NO concentrations, casting doubt on its physiological relevance. In this paper we present evidence that at submicromolar NO concentrations the aerobic nitrosation of glutathione does not involve NO autoxidation but a reaction that is first order in NO. We show that this reaction produces nitrosoglutathione efficiently in a reaction that is strongly stimulated by physiological concentrations of Mg^{2+}. These observations suggest that direct aerobic nitrosation may represent a physiologically relevant pathway of nitrosothiol formation.

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Nitric oxide (NO) exhibits a vast range of functions in signal transduction and the immune response in mammalian tissues [1]. Many functions in signal transduction are mediated by the NO-sensitive (soluble) isoform of guanylate cyclase [2]. However, under some conditions NO is converted to compounds with distinct properties that may alter its (patho)physiological impact [3].

Nitrosothiols are endogenously occurring formal adducts of protein or low-molecular-weight thiols with the one-electron oxidized form of nitric oxide, NO^+ [4]. Evidence is mounting that nitrosothiols may perform distinct functions in biology [5,6]. Because nitrosothiols release NO under certain conditions and are generally more stable than NO, they may function as a storage and transport pool of NO. Nitrosothiols also exhibit biological actions completely different from those of NO, because nitrosation of specific cysteine residues may alter protein function. Hence, S-nitrosation is increasingly regarded as a posttranslational modification akin to phosphorylation [7–9].

Despite these potential (patho)physiological ramifications there is no consensus about the way in which nitrosothiols are generated cellularly [8,10–13]. In the laboratory nitrosothiols are synthesized at low pH from the corresponding thiols and nitrous acid. Biologically, similar reactions may occur under special conditions, such as in the stomach or in activated macrophages. At physiological pH nonenzymatic formation of nitrosothiols may be catalyzed by transition metals such as copper ions [14]. In view of the extremely low in vivo free copper concentration and the reversible nature of the reaction—copper ions also catalyze the decomposition of nitrosothiols [4,8,15]—the physiological relevance of this reaction is questionable. Nitrosothiols can also be formed by decomposition of low-molecular-weight dinitrosyl–iron complexes (DNICs) with thiolate ligands [16,17]. As DNICs are formed in vivo, these compounds constitute serious candidates as biological nitrosating agents. However, because the mechanism of biological DNIC formation has not been elucidated and because DNICs may also catalyze nitrosothiol breakdown, the exact role of DNICs in biological nitrosothiol formation is unclear. In addition to these nonenzymatic processes, several enzymes have been implicated in nitrosothiol formation, such as the copper protein ceruloplasmin [18] or hemoproteins [8,19]. Nitrosothiols will also be formed when NO and O_2^- are cogenerated at similar rates [11,20], which may be physiologically relevant under some conditions, for instance, when partly uncoupled nitric oxide synthase generates NO and O_2^- simultaneously [21].

The best studied pathway of nitrosothiol formation is the aerobic reaction of NO with glutathione (GSH). Anaerobically, NO does not form *S*-nitrosoglutathione (GSNO), but in the presence of oxygen GSNO is generated in a reaction that is first order in O_2 and second order in NO [22–24]. The reaction starts with the

Abbreviations:: GSNO, S-nitrosoglutathione; DTPA, diethylenetriaminepentaacetic acid; DEA/NO, diethylamine NONOate (diethylammonium (*Z*)-1-(*N,N*-diethylamino)diazen-1-ium-1,2-diolate); PROLI/NO, proline NONOate (1-(hydroxyl-*N,N,O*-azoxy)-l-proline, disodium salt); DNIC, dinitrosyl–iron complex; SOD, superoxide dismutase; oxy-Hb, oxyhemoglobin; met-Hb, methemoglobin; TEA, triethanolamine; DAN, 2,3-diaminonaphthalene; DTT, dithiothreitol; NAC, *N*-acetylcysteine; β-ME, β-mercaptoethanol.

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.04.034

rate-limiting formation of NO_2 from NO and O_2 (Eq. (1)). According to one hypothesis [23,25], NO_2 then reacts with another molecule of NO to form the strong nitrosating agent N_2O_3, which reacts with GSH to GSNO and nitrite (Eqs. (2) and (3)). Alternatively, it has been proposed [11,24,26] that NO_2 reacts directly with GSH to produce nitrite and a glutathiyl radical ($GS^{\bullet}$) that instantaneously combines with NO to GSNO (Eqs. (4) and (5)):

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO_2 + NO \leftrightarrow N_2O_3 \quad (2)$$

$$N_2O_3 + GSH \rightarrow GSNO + H^+ + NO_2^- \quad (3)$$

$$NO_2 + GSH \rightarrow GS^{\bullet} + H^+ + NO_2^- \quad (4)$$

$$NO + GS^{\bullet} \rightarrow GSNO \quad (5)$$

Because of the low (submicromolar) physiological concentrations of NO, this pathway is expected to be too slow to make an impact. In 1997 it was reported that, for low NO concentrations (≤1 μM), aerobic nitrosothiol formation was not due to NO autoxidation but involved a direct reaction between NO and the thiol [27]. However, later studies could not confirm that mechanism [17,28]. In the present study we demonstrate that the aerobic nitrosation of glutathione by submicromolar NO is first order in NO and proceeds more efficiently than previously thought, making it a serious candidate as a participant in nitrosothiol formation in vivo after all.

Materials and methods

All reagents were obtained from Merck (Vienna, Austria) or Sigma (Vienna, Austria), except for diethylamine NONOate (DEA/NO), proline NONOate (PROLI/NO), and GSNO, which were purchased from Enzo Life Sciences (Lausen, Switzerland). Stock solutions were made in ultrapure water (Barnstead, resistance $> 18\ M\Omega\ cm^{-1}$), except for DEA/NO and PROLI/NO, which were dissolved in 10 mM NaOH; GSH, which was dissolved in 1 M NaOH; and GSNO, which was dissolved in 10 mM HCl.

Determination of NO and nitrosothiols with the NO-sensitive electrode

Nitric oxide was measured with a Clark-type electrode from Iso-NO (WPI, Berlin, Germany) according to a published method [29]. Unless indicated otherwise, experiments were performed in open stirred vessels. Reactions were started by the introduction of DEA/NO (or PROLI/NO, 1 μM) in a total volume of 0.5 ml of 50 mM triethanolamine (TEA) buffer (pH 7.4) and GSH (routinely 1 or 2 mM), 5 mM $MgCl_2$, 1000 U/ml superoxide dismutase (SOD), and 0.1 mM diethylenetriaminepentaacetic acid (DTPA). After complete decay of the NO signal, $CuSO_4$ (4 mM) was added to measure nitrosothiol formation [30]. When indicated, $MgCl_2$ was omitted or replaced by $CaCl_2$, $MnCl_2$, $ZnCl_2$, or NaCl. Some experiments were performed in 50 mM potassium phosphate (KP_i) or Tris–HCl instead of TEA and some experiments were performed in the presence of 1 mM NAD^+ or NADH. Dependence of the NO curves on the concentration of DEA/NO was investigated between 10 nM and 1 μM. The effect of the GSH concentration was studied between 1 μM and 5 mM. At the highest GSH concentration, we increased the $CuSO_4$ concentration to 10 mM, because copper ion-induced NO release from GSNO is slow in the presence of excess GSH [15].

Some experiments were performed in closed vessels with reaction volumes of 0.5 or 1.8 ml. Completely filled closed vessels were also used to determine the effect of O_2. For these experiments solutions were bubbled with argon before use. Reaction mixtures were then incubated in septum-sealed, completely filled vessels and bubbled with argon for 15 min. Subsequently, the gas supply line was removed and experiments were started by addition of the NO donor. Because the covering of the sample under these conditions is not completely airtight, slow readmission of air into the sample occurs.

Calibration of the electrode was performed daily with $NaNO_2$/KI [29]. Pre- and post-Cu^{2+} peaks are presented as micromolar NO based on those calibrations. To quantify the concentration of GSNO detected by $CuSO_4$ addition, calibration curves were determined with authentic GSNO in concentrations between 0.1 and 2.0 μM. GSNO stock solutions were prepared in 10 mM HCl and used immediately. The concentration of the stock solutions was checked spectrophotometrically at 340 nm after 10-fold dilution in 50 mM KP_i (pH 7.4). Calibration curves were linear over the full concentration range ($R = 0.999$).

Quantification of NO released by DEA/NO

To determine how much NO is released by DEA/NO under the present experimental conditions, we measured the conversion of oxyhemoglobin (oxy-Hb) to methemoglobin (met-Hb) spectrophotometrically from the absorbance difference between 420 and 401 nm according to a published procedure [31], but with 50 mM TEA (pH 7.4) instead of KP_i as the buffer.

Determination of NO, GSNO, and nitrite with the NO analyzer

A nitric oxide analyzer NOA 280 (Sievert Instruments, USA) was used to determine the amounts of NO, GSNO, and nitrite by chemiluminescence detection according to a published method [32]. Briefly, samples (500 μl) were injected in a purging vessel filled with KI/I_2 (45 mM/10 mM) in glacial acetic acid. Under these conditions both nitrite and GSNO are reduced to NO. In parallel, a second set of samples was incubated with 10% of a solution of sulfanilamide (5% in 1 N HCl) for 1 min to scavenge nitrite and measure only the remaining GSNO. Calibration curves with authentic GSNO (0.1–2 μM) and nitrite (0.1–1 μM) were measured daily in sample buffer.

Alternatively, GSNO and GSNO+nitrite were measured by addition of 4 mM $CuSO_4$ and 4 mM $CuSO_4$+KI/I_2 (45 mM/10 mM), respectively, whereas NO was determined in the absence of these substances.

Fluorimetric determination of nitrite in the presence of 2,3-diaminonaphthalene

Nitrite was determined using 12.6 μM 2,3-diaminonaphthalene (DAN) to form the fluorescent product 1-(*H*)-naphthotriazole [33]. Briefly, samples were incubated for 20 min with DAN under acidic conditions. After stabilization with 1 N sodium hydroxide, formation of 1-(*H*)-naphthotriazole was measured using an LS50B luminescence spectrometer (PerkinElmer, UK) with excitation and emission at 365 and 425 nm, respectively. Alternatively, a commercial assay (Nitrite/Nitrate Assay Kit, Cat. No. 06239; Sigma–Aldrich) based on the same principle was used. Calibration curves were measured daily with authentic nitrite (0.1–1 μM) in sample buffer.

Determination of nitrite, nitrate, and GSNO by HPLC

In some experiments we increased the sensitivity by separating the reaction products by HPLC (Merck–Hitachi D-6000; Vienna, Austria). For nitrite/nitrate determination the same commercial nitrite/nitrate-kit described above was used according to the

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