Free Radical Biology & Medicine 47 (2009) 458–467

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Chemical model systems for cellular nitros(yl)ation reactions

Andreas Daiber [a,*,1], Stefan Schildknecht [b,1], Johanna Müller [a], Jens Kamuf [a], Markus M. Bachschmid [c], Volker Ullrich [b]

[a] Second Medical Clinic, Department of Cardiology, Johannes Gutenberg University, 55101 Mainz, Germany
[b] Department of Biology, University of Konstanz, Germany
[c] Department of Medicine, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118, USA

ARTICLE INFO

Article history:
Received 27 August 2008
Revised 21 April 2009
Accepted 18 May 2009
Available online 27 May 2009

Keywords:
Nitric oxide
Superoxide
Peroxynitrite
Nitrosation reactions
S-nitrosylation
Nitrosophenol
Diaminonaphthalene
Free radicals

ABSTRACT

S-nitros(yl)ation belongs to the redox-based posttranslational modifications of proteins but the underlying chemistry is controversial. In contrast to current concepts involving the autoxidation of nitric oxide ($^{\bullet}NO$, nitrogen monoxide), we and others have proposed the formation of peroxynitrite (oxoperoxonitrate (1−)) as an essential intermediate. This requires low cellular fluxes of $^{\bullet}NO$ and superoxide ($^{\bullet}O_2^-$), for which model systems have been introduced. We here propose two new systems for nitros(yl)ation that avoid the shortcomings of previous models. Based on the thermal decomposition of 3-morpholinosydnonimine, equal fluxes of $^{\bullet}NO$ and $^{\bullet}O_2^-$ were generated and modulated by the addition of $^{\bullet}NO$ donors or Cu,Zn-superoxide dismutase. As reactants for S-nitros(yl)ation, $NADP^+$-dependent isocitrate dehydrogenase and glutathione were employed, for which optimal S-nitros(yl)ation was observed at nanomolar fluxes of $^{\bullet}NO$ and $^{\bullet}O_2^-$ at a ratio of about 3:1. The previously used reactants phenol and diaminonaphthalene (C- and N-nitrosation) demonstrated potential participation of multiple pathways for nitros(yl)ation. According to our data, neither peroxynitrite nor autoxidation of $^{\bullet}NO$ was as efficient as the 3 $^{\bullet}NO$/1 $^{\bullet}O_2^-$ system in mediating S-nitros(yl)ation. In theory this could lead to an elusive nitrosonium (nitrosyl cation)-like species in the first step and to N_2O_3 in the subsequent reaction. Which of these two species or whether both together will participate in biological S-nitros(yl)ation remains to be elucidated. Finally, we developed several hypothetical scenarios to which the described $^{\bullet}NO/^{\bullet}O_2^-$ flux model could apply, providing conditions that allow either direct electrophilic substitution at a thiolate or S-nitros(yl)ation via transnitrosation from *S*-nitrosoglutathione.

Redox regulation comprises several chemical mechanisms of oxidative or reductive modifications of proteins involved in the regulation of metabolic or biosynthetic pathways. These modifications include zinc-finger oxidation, methionine sulfoxidation, and oxidation or nitration of tyrosine residues [1], which all can be assigned to peroxynitrite derived from equal fluxes of nitric oxide (nitrogen monoxide, $^{\bullet}NO$) and superoxide anion ($^{\bullet}O_2^-$). The high reactivity of peroxynitrite ($ONOO^-$) allows such posttranslational modifications to occur in the submicromolar range and hence under physiological conditions [2,3].

An abundant posttranslational modification is S-nitros(yl)ation[2] of Cys residues in proteins and low-molecular-weight thiols such as glutathione. Although S-nitros(yl)ation is abundant under physiological and pathophysiological conditions [4–7], neither are the underlying mechanisms well understood nor is there a unifying hypothesis on its physiological significance [8]. Because many enzymes rely on thiols as essential catalytic or structural groups, an inhibition but also an activation could be a consequence, as exemplified by the regulation of the NMDA receptor [9], HIF [10], or NF-κB [11], which have all been reported to involve S-nitros(yl)ation. According to the pioneering work of Stamler et al. [12,13], it can be assumed that enhanced biosynthesis of $^{\bullet}NO$ during cellular activation leads to a distinct nitros(yl)ation pattern in which glutathione (GSH) may be a primary target of a still elusive nitrosating agent [14]. By a mechanism of transnitros

Abbreviations: Cu,Zn-SOD, copper, zinc superoxide dismutase; DAN, diaminonaphthalene; DHR, dihydrorhodamine 123; GSH, glutathione; GSNO, *S*-nitrosoglutathione; HX, hypoxanthine; ICDH, isocitrate dehydrogenase; PEG-SOD, polyethylene glycolated Cu,Zn-SOD; Sin-1, 3-morpholino sydnonimine; SPENO, spermine NONOate; XO, xanthine oxidase.

* Corresponding author. Fax: +49 (0)6131 3933304.
E-mail address: andreas.daiber@bioredox.com (A. Daiber).

[1] These authors contributed equally to this work.

[2] With the nomenclature "S-nitros(yl)ation" that we use throughout the article, we conform with the numerous publications on biological S-nitros(yl)ation. However, this term does not reflect the important difference between "nitrosylation" caused by addition of $^{\bullet}NO$ to metal centers and "nitrosation" at a nucleophilic carbon, nitrogen, or sulfur atom leading to formal esters of nitrous acid.

0891-5849/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2009.05.019

(yl)ation, other proteins containing consensus sequences for *S*-nitrosoglutathione (GSNO) binding may follow [15]. This hypothesis is supported by the high pK_a value of GSH and hence a high group transfer potential of GSNO to thiols or thiolates of proteins with low pK_a values. N-nitrosation has also been observed under cellular conditions, thus allowing the conclusion that an elusive nitrosating species exhibits a broad target spectrum [16–19]. However, there are no literature data available on the physiological relevance of N-nitroso species.

Formally, nitros(yl)ation proceeds by the reaction of a nitrosonium ion (NO^+) with a nucleophilic group (Eq. (1)), but it is generally understood that in a physiological pH range, NO^+ is not stable but immediately forms nitrite (dioxonitrate(1−)) (Eq. (1a)):

$$R - XH + \left[NO^+\right] \rightarrow R - X - NO + H^+, \quad (1)$$

$$\left[NO^+\right] + OH^- \rightarrow NO_2^- + H^+. \quad (1a)$$

However, dinitrogen trioxide (N_2O_3), the anhydride of nitrous acid, is a well-known nitrosating agent that can be formed from $^{\bullet}NO$ and the $^{\bullet}NO_2$ radical (Eq. (3)). The latter may arise as an autoxidation product of $^{\bullet}NO$ in the presence of dioxygen (Eq. (2)), but this reaction is second order with regard to $^{\bullet}NO$ and hence becomes very slow at physiological concentrations of $^{\bullet}NO$ [20]. Therefore the autoxidation of $^{\bullet}NO$ with subsequent formation of N_2O_3 seems to be an implausible model for biological S-nitros(yl)ation:

$$2^{\bullet}NO + O_2 \rightarrow 2^{\bullet}NO_2, \quad (2)$$

$$^{\bullet}NO_2 + {}^{\bullet}NO \rightarrow N_2O_3. \quad (3)$$

This also renders the alternative mechanism of electron abstraction from a thiolate by $^{\bullet}NO_2$ or $^{\bullet}O_2^-$ unlikely [21]. Recent reports showing thiyl radical trapping in the presence of peroxynitrite [22] used high concentrations of peroxynitrite in the presence of CO_2, which easily leads to homolytic cleavage [23] of the CO_2/peroxynitrite adduct. It has been pointed out repeatedly that bolus additions of peroxynitrite may give erroneous results compared to the generation of peroxynitrite by low fluxes of $^{\bullet}NO$ and $^{\bullet}O_2^-$ as seen under physiological conditions [24]. However, recent kinetic data were in favor of thiyl-mediated S-nitros(yl)ation, requesting consideration of this pathway as well [25].

A second pathway leading to an NO^+-like species would be catalysis of $^{\bullet}NO$ oxidation by an oxidized transition metal complex as present in nitroprusside ($Fe(III)NO(CN)_5)^{2-}$. This would require continuous reoxidation of the reduced metal ion. Indeed, metal-based mechanisms for nitros(yl)ation were previously proposed [26,27].

More recently, a third pathway was suggested when indirect evidence indicated an interaction of $^{\bullet}NO$ with peroxynitrous acid [16,28,29], which owing to its pK_a value of 6.6, is in equilibrium with peroxynitrite [30]. According to the reaction

$$^{\bullet}NO + HOONO \rightarrow NO_2^- + H^+ + {}^{\bullet}NO_2, \quad (4)$$

the $^{\bullet}NO_2$ radical could yield N_2O_3 with an additional molecule of $^{\bullet}NO$, resulting eventually in a stoichiometry of 3 $^{\bullet}NO$ to 1 $^{\bullet}O_2^-$ for the nitros(yl)ation process via N_2O_3. If the above reaction would be sufficiently fast, a low steady-state level of peroxynitrite would be the consequence in the presence of an excess of $^{\bullet}NO$. This was experimentally supported by complete abolishment of the peroxynitrite-mediated zinc-finger oxidation in alcohol dehydrogenase at an about threefold molar excess of $^{\bullet}NO$ over $^{\bullet}O_2^-$ [29]. Superoxide fluxes were generated by hypoxanthine/xanthine oxidase (HX/XO) and $^{\bullet}NO$ fluxes by spermine NONOate (SPENO). Whereas Espey et al. [16] observed an optimum of diaminonaphthalene (DAN) nitrosation also at a ratio of 3 $^{\bullet}NO$:1 $^{\bullet}O_2^-$, we had reported nitrosation of phenol by co-incubating equimolar concentrations of 3-morpholinosydnonimine hydrochloride (Sin-1) and SPENO [29]. The aerobic thermal decomposition of Sin-1 releases $^{\bullet}NO$ and $^{\bullet}O_2^-$ at equal rates [31] and SPENO generates 2 mol of $^{\bullet}NO$ [32], thus resulting in a stoichiometric release of 3 $^{\bullet}NO$ and 1 $^{\bullet}O_2^-$. This system yielded fivefold higher amounts of 4-nitrosophenol compared to Sin-1 and SPENO alone [29]. To address the underlying mechanism in detail we here used the methodology and the concept of two papers published in 2002 [16,29].

Precise kinetics of the decay curves, however, were not considered in these previous publications. In such complex systems, secondary reactions of NONOate with $^{\bullet}O_2^-$ or peroxynitrite could arise, or the kinetics of $^{\bullet}O_2^-$ generation by xanthine oxidase could have been modified by reactive intermediates. In addition, because peroxynitrite at pH 12 did not react with $^{\bullet}NO$, the reaction of $^{\bullet}NO$ with peroxynitrous acid (Eq. (4)) also has been seriously questioned [33].

To highlight the biological significance of the herein described nitros(yl)ation mechanism, $NADP^+$-dependent isocitrate dehydrogenase (ICDH) was chosen as a biologically relevant target, because its S-nitros(yl)ation had been shown to inhibit the enzyme [34,35]. However, it should be noted that purified enzymes are only part of a biological model because the complex environment of the cell is only partially mimicked [36].

As a new nitros(yl)ation model we here propose the aerobic decay of Sin-1 in the presence of Cu,Zn-superoxide dismutase (Cu,Zn-SOD), which was suited to display S-nitros(yl)ation of ICDH at a defined maximum without considerable S–NO formation by peroxynitrite or $^{\bullet}NO$ alone. This system has the potential to serve as a suitable model to study cellular S-nitros(yl)ation reactions under kinetically identical flux conditions for $^{\bullet}NO$ and $^{\bullet}O_2^-$.

Materials and methods

Materials

Sin-1 was obtained from Calbiochem (La Jolla, CA, USA). SPENO was purchased from Cayman Chemicals (Ann Arbor, MI, USA), DAN and dihydrorhodamine 123 (DHR) were from Fluka (Buchs, Switzerland). Polyethylene-glycolated Cu,Zn-superoxide dismutase (PEG-SOD; EC 1.15.1.1) from bovine erythrocytes, $NADP^+$-dependent ICDH (EC 1.1.1.42) type IV from porcine heart (solution in 50% glycerol), XO (EC 1.1.3.22) grade III from buttermilk, cytochrome *c, S*-nitrosoglutathione, and 4-nitrosophenol were provided by Sigma–Aldrich (Deisenhofen, Germany).

N-nitrosation

A solution of DAN (2.5, 20, or 100 μM) in potassium phosphate buffer (0.1 M, pH 7.4) was incubated with a fixed concentration of Sin-1 (1, 10, or 100 μM) in the presence of increasing amounts of SPENO (0.1–1000 μM) for 90 min at 37°C. $^{\bullet}NO$-alone control was performed with 20 μM DAN and SPENO (1–100 μM) without Sin-1. DAN N-nitrosation (triazol formation) was measured by fluorescence detection (excitation 370 nm; emission 460 nm) using a Twinkle fluorescence plate reader (Berthold Technologies, Bad Wildbad, Germany). Similar experiments were performed with a solution of DAN (20 or 100 μM) and a fixed concentration of Sin-1 (10 or 100 μM) in the presence of increasing amounts of PEG-SOD (0.00033–100 U/ml) for 90 min at 37°C. $^{\bullet}NO$-alone control was performed with 20 μM DAN and 10 μM SPENO with PEG-SOD (0.00033–100 U/ml). The effect of bicarbonate (25 mM) on N-nitrosation was studied in a system with 20 μM DAN, 10 μM Sin-1, and PEG-SOD (0.00033–100 U/ml). The concentration-dependent effects of bicarbonate (0.001–100 mM), uric acid (0.0001–10 mM), and sodium azide (0.001–100 mM) were tested under maximal nitrosation conditions in the presence of 100 μM Sin-1