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Similarity and dissimilarity of thiols as anti-nitrosative agents in the nitric oxide–superoxide system

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ABSTRACT

Concomitant production of nitric oxide and superoxide in biological systems has been proposed to generate numerous reactive oxygen and nitrogen species that cause oxidative and nitrosative stress. Thiols, especially glutathione, play an important role in cellular defense against radical species. In the present study, we investigated and compared the anti-nitrosative activity of a wide range of thiols in a simplified chemical system of co-generated nitric oxide and superoxide. Of the 13 thiols studied, three groups of thiols are distinguishable: (i) Group I includes cysteine and its four congeners (cysteine methyl ester, cysteine ethyl ester, homocysteine, cysteamine); they are subject to rapid oxidative decomposition and have the least anti-nitrosative activity. (ii) Group II consists of glutathione, penicillamine, tiopronin and mesna; they have the greatest effect on delaying the nitrosation reaction. (iii) Group III comprises *N*-acetylcysteine, *N*-acetylpenicillamine, captopril, and thioglycolate; they all have high pK_a for the mercapto group and show the strongest inhibitory effect on the rate and extent of nitrosation in the system studied.

1. Introduction

Simultaneous presence of nitric oxide and superoxide has been implicated in many pathological conditions, including ischemia–reperfusion and inflammation [1]. While a high flux of nitric oxide is mainly generated by inducible nitric oxide synthase, superoxide is produced from a variety of sources, such as NADPH oxidase, xanthine oxidase, and the mitochondrial electron transport chain [1]. Nitric oxide rapidly reacts with superoxide to form the potent oxidant peroxynitrite, and the nitric oxide–superoxide–peroxynitrite triad forms a complex chemical system of biological significance. Chemically, the triad system would generate multiple reactive oxygen species (ROS) and reactive nitrogen species (RNS) as a result of subsequent decomposition and reaction chemistry of peroxynitrite [2]. Biologically, imbalance between ROS and RNS may result in cellular stress (oxidative, nitroxidative, nitrosative, nitrative), thereby mediating redox signaling and disease states [3–8].

The chemistry of a system of co-generated nitric oxide and superoxide has been extensively studied. In general, most previous studies have focused on investigating one of the three major reactions, i.e. oxidation, nitration, and nitrosation, in the context of varying fluxes of both radicals. Miles et al. [9] have shown that dihydrorhodamine oxidations were maximal at equivalent fluxes of nitric oxide and superoxide, and excess production of either radical remarkably inhibits this oxidation reaction (i.e. a bell-shaped response curve). In addition, Wink et al. [10] have shown that GSH oxidation was peaking when the rates of formation of both radicals were at a ratio of near 1:1. Furthermore, the characteristic bell-shaped response curve has also been observed in nitration and nitrosation reactions derived from the nitric oxide–superoxide reactions [11–13]. Recently, the study of Daiber et al. [14] has demonstrated that nitrosation reactions (*C*-, *N*-, and *S*-nitrosation) occur maximally at a 3:1 nitric oxide–superoxide flux ratio. More recently, using a specific probe for detecting peroxynitrite, Zielonka et al. [15] showed that peroxynitrite was the major species formed in the nitric oxide–superoxide co-generation system and the response curve was nevertheless reaching a plateau at a 1:1 ratio. The discrepancy among different experimental models highlights the complex, dynamic nature of nitric oxide–superoxide interaction [16].

Thiol chemistry is an integral part of redox biology [1,17,18]. On the one hand, cysteine modification of cellular thiol proteins has been recognized as a pivotal mechanism underlying redox signaling [1,18]; on the other, glutathione (GSH) and small-molecule thiol compounds could act as effective antioxidants that protect cells from free radical injury [17]. Thiols react with almost all physiological oxidants, and, in the nitric oxide–superoxide system, two types of oxidation reactions may occur simultaneously, i.e.

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione (reduced form); SIN-1, 3-morpholinosydnonimine HCl; DAN, 2,3-diaminonaphthalene; DTPA, diethyltriaminepentaacetic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DMSO, dimethyl sulfoxide.

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one-electron vs. two-electron oxidation [1]. In such a system, thiols react with free radical targets, including superoxide, $\cdot NO_2$, $CO_3^{\cdot-}$ and $\cdot OH$, via one-electron reaction, and generate the thiyl radical, whereas direct thiol-peroxynitrite reaction involves two-electron oxidation [1,19]. Furthermore, thiols react with nitric oxide [20,21] and its oxidation product, N_2O_3 [22–25].

Rate constants for thiols reacting with a variety of ROS–RNS have been reported, and the values span several orders of magnitude [26–32]. For example, rate constants for the thiol-superoxide reaction are usually $<10^3\ M^{-1}\ s^{-1}$ [27,31,32], whereas those for the thiol-peroxynitrite [30] and for the thiol-$\cdot NO_2$ reaction [26] are around 10^5 and $10^7\ M^{-1}\ s^{-1}$, respectively. Moreover, the reactivity of thiols reacting with a single ROS–RNS (e.g. superoxide or peroxynitrite) has been compared among thiols and the results showed that the reactivity depends on thiol pK_a [30,32]. In physiologically relevant conditions, however, thiols would react with multiple ROS–RNS, and the collective outcome of these reactions may not be determined by a single factor or reaction. It was therefore of interest to study the overall effect of thiols as modulating agents for nitroxidative stress. In the present study, we have investigated and compared the capability of 13 different thiols as anti-nitrosative agents in a system of co-generated nitric oxide and superoxide.

2. Materials and methods

2.1. Materials

SIN-1 (3-morpholinosydnonimine HCl), 2,3-diaminonaphthalene (DAN), diethyltriaminepentaacetic acid (DTPA), catalase, glutathione, cysteine, cysteamine, homocysteine, cysteine methyl ester, cysteine ethyl ester, *N*-acetylcysteine, penicillamine, ammonium thioglycolate, captopril, *N*-acetylpenicillamine, mesna (sodium 2-mercaptoethanesulfonate), tiopronin (*N*-(2-mercaptopropionyl)glycine), and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Nitrosation kinetics [33]

SIN-1 mediated DAN nitrosation was kinetically monitored by a fluorometric microplate reader (Infinite M200, Tecan Austria GmbH). Reactions were performed in 96-well microplates (Nunc, Denmark) at 37 °C. The reaction buffer consists of DAN (3.15 μM), DTPA (0.1 mM), catalase (120 U/ml) and phosphate-buffered saline (pH 7.4; 10 mM phosphate buffer containing 138 mM NaCl and 2.7 mM KCl). Reactions were initiated by adding 6 μL SIN-1 (5 mM stock solution in DMSO) into 294 μL reaction buffers containing various concentrations of thiol compounds. The fluorescence intensity (excitation: 380 nm; emission: 460 nm; gain = 100) after the addition of SIN-1 (100 μM) was measured at 10-min intervals.

2.3. Thiol decomposition kinetics

The decomposition kinetics of thiol compounds (drugs) in the SIN-1 reaction system was determined in the same 96-well format. At each predetermined sampling time (every 5–10 min), 250 μL of sample was withdrawn from each well and immediately added to 350 μL assay solution containing 200 μM DTNB (Ellman's reagent). Spectrophotometric measurements were then performed at 412 nm [34].

2.4. Kinetic simulation

A kinetic model was built by integrating the system of GSH reactions with the base model of the SIN-1 reaction system [33] (Supplementary Table S1). Overall, the complete GSH model encompasses more than 45 chemical reactions and rate constants. The majority of the rate constants used was derived from published data. The rate of change in the concentration of each chemical species is expressed as an ordinary differential equation, based on the law of mass action. Kinetic simulations were performed by numerically solving the differential equations (Mathematica 6, Wolfram Research, Champagne, IL). The proposed kinetic model can adequately describe the nitrosation kinetics mediated by GSH as well as the decomposition kinetics of GSH in the system studied (Supplementary Fig. S1).

2.5. Data analysis

Experiments reported herein were performed three times, with similar results being obtained. Statistical analysis of data was made with a Student's *t*-test. Changes were considered statistically significant when $p < 0.05$.

3. Results

Fig. 1 shows the kinetic profiles of SIN-1-mediated *N*-nitrosation in the absence and presence of various concentrations (2–100 μM) of GSH. Clearly, increasing GSH concentrations resulted in right shift of the kinetic profiles (Fig. 1A), with increasing lag times (Fig. 1B) and decreasing slopes (of the acceleration phase) and peaks (maximum intensity, Fig. 1C). For the purpose of comparison, similar experiments and analyses have been conducted for additional 12 thiols. The results show that the 13 thiols studied can be categorized into three groups, based on their distinctive effect on the slope and maximum intensity of the kinetic profile (Fig. 2A and B). Group I contains five thiols, including cysteine and its congeners (cysteamine, homocysteine, cysteine methyl ester, cysteine ethyl ester), which has little effect on both the slope (Fig. 2A) and the maximum intensity (Fig. 2B). Furthermore, Group II thiols include GSH and three therapeutic thiols (mesna, penicillamine, tiopronin), which, compared to the Group I thiols, exert much stronger inhibitory effect on both parameters. Finally, the third group of thiols comprise another four therapeutic thiols (captopril, *N*-acetylcysteine, thioglycolate, *N*-acetylpenicillamine), which nevertheless show the strongest inhibitory effect on both parameters (Fig. 2A and B). When the lag-time-prolonging effect of thiols was compared, Group II thiols were shown to be the most effective, whereas Group I and III thiols are indistinguishable in this regard (Fig. 2C).

The disparity–similarity among thiols can be further revealed by a set of parametric plots, as shown in Supplementary Fig. S2: first, in the slope vs. maximum intensity plot, data for various thiols of distinct groups are about a straight line (Supplementary Fig. S2A); secondly, although data are scattered in different regions of the slope vs. lag time plot, data for Group I and II thiols appear to be correlated (Supplementary Fig. S2B); thirdly, Group III thiols are unique in that they have high pK_a values, i.e. p$K_{a,SH} > 9.5$ (Supplementary Fig. S2C).

The decomposition kinetics of GSH in the SIN-1 system has been determined at 4 different GSH levels and compared with the nitrosation kinetics. The results in Fig. 3 show that concentrations of reduced GSH continued to decline during the lag phase of nitrosation; only after GSH levels were reaching an insignificant level did the nitrosation reaction proceed. Thus, for GSH, there exists an apparent association between the lag time of nitrosation and the time to complete GSH decay (Fig. 3B). Similarly, decomposition kinetics of other thiols at a fixed initial thiol concentration (20 μM) has also been measured. The results indicate that Group I and II (including GSH) thiols behave similarly because, for each group, the lag time and the time to thiol depletion are comparable (Fig. 4). Group III thiols, however, exhibit a totally different kinetic pattern – the average time to complete thiol decomposition