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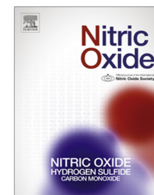

The dual effects of nitrite on hemoglobin-dependent redox reactions

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ABSTRACT

Evidence to support the role of heme proteins-dependent reactions as major inducers of oxidative damage is increasingly present. Nitrite (NO_2^-) is one of the major end products of NO metabolism, and from the daily consumption. Although the biological significance of heme proteins/NO_2^--mediated protein tyrosine nitration is a subject of great interest, the important roles of NO_2^- on heme proteins-dependent redox reactions have been greatly underestimated. In this study, we investigated the influence of NO_2^- on met-hemoglobin (Hb)-dependent oxidative and nitrative stress. It was found that NO_2^- effectively reduced cytotoxic ferryl intermediate back to ferric Hb in a biphasic kinetic reaction. However, the presence of NO_2^- surprisingly exerted pro-oxidant effect on Hb–H_2O_2-induced protein (bovine serum albumin, enolase) oxidation at low concentrations and enhanced the loss of HepG2 cell viability. In the reduction of ferryl Hb to ferric state, NO_2^- was decreased and oxidized to a nitrating agent NO_2, Tyr12 and Tyr191 in enolase were subsequently nitrated. In contrast to the frequently inhibitive effect of nitrotyrosine, NO_2^--triggered tyrosine nitration might play an important role in enolase activation. These data provided novel evidence that the dietary intake and potential therapeutic application of NO_2^- would possess anti- and pro-oxidant activities through interfering in hemoglobin-dependent redox reactions. Besides the classic role in protein tyrosine nitration, the dual effects on hemoglobin-triggered oxidative stress may provide new insights into the physiological and toxicological implications of NO_2^- with heme proteins.

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1. Introduction

Heme proteins carry out a myriad of diverse biological functions, such as oxygen storage, transport and reduction, electron transfer and redox catalysis [1]. On the other hand, both intact and degraded forms of heme proteins are efficient inducers of oxidative damage [1–3]. Recent research has shown that myoglobin (Mb) and hemoglobin (Hb) play important roles in the pathophysiology of certain disease states, such as vasospasm following subarachnoid hemorrhages and renal dysfunction following rhabdomyolysis [4,5]. These pathologies are linked to the interaction of heme group with peroxides to initiate oxidative reactions, including the formation of free radical species and hypervalent states of the heme iron that attack cellular components [4–8].

Nitrite (NO_2^-) is one of the major end products of nitric oxide (NO) metabolism, and from the daily consumption [9,10]. It has been found that levels of NO_2^- in healthy human plasma is about 350 nM, and the decrease in plasma nitrite levels reflects the endothelial dysfunction in humans and is correlated with increasing cardiovascular risk load [9]. NO_2^- has been used for decades in the food industry as a preservative and for curing meat. Furthermore, dietary NO_2^- supplementation has been proposed as an oral and gut antimicrobial agent [11,12]. Nitrite has recently emerged as an endogenous signaling molecule with potential therapeutic implications for cardiovascular disease. It is now clear that NO_2^- can act as an anti-oxidant and a promising therapeutic agent to protect against myocardial ischemia-reperfusion injury through mediating NO homeostasis [13,14]. Moreover, NO_2^- accumulation at inflammatory sites may be cytoprotective against damage caused by HOCl [11,12].

In contrast to the protective properties of NO_2^- in cell and tissue injuries, NO_2^- dramatically enhances H_2O_2 toxicity in the presence

Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenol; DNPH, 2,4-dinitrophenylhydrazine; Hb, hemoglobin; MPO, myeloperoxidase; Mb, myoglobin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 3-NT, 3-nitrotyrosine.

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of heme proteins in vitro [15–17]. It has been investigated that the increased toxicity is due to the formation of free NO_2 from the reaction of NO_2^- with the hypervalent form of heme proteins. Subsequently, 3-nitrotyrosine residues are formed in the reaction between NO_2 and tyrosyl radical [15,17]. Therefore, the heme proteins-catalyzed oxidation of NO_2^- has been proposed to represent an alternative source of tyrosine nitration [10,15]. In addition to $ONOO^-$-mediated nitration, heme proteins/NO_2^--catalyzed tyrosine nitration may also contribute to cell and tissue injury under conditions of increased NO production. The nitration of proteins modulates catalytic activity, cell signaling and cytoskeletal organization [18]. Meanwhile, extensive evidence supports that the formation of nitrotyrosine has been used as a biomarker of NO-mediated pathological disease and oxidative stress process [10,15–19]. The biological significance of tyrosine nitration, therefore, is a subject of great interest.

However, the important roles of NO_2^- on heme proteins-dependent redox reactions were greatly underestimated. During the nitration of proteins, oxidative damage of protein is always accompanied and characterized by the formation of protein carbonyl group which has been widely used as a biomarkers of oxidative stress [20,21]. The effect of this classic inorganic compound on heme proteins-dependent redox reactions was mainly focused on the formation of protein tyrosine nitration [10,15–19], there were few data on the effect of NO_2^- on heme proteins-dependent protein oxidation, and potential different effects of oxidative and nitrative modifications on protein function. With the potential biological significance of NO_2^-, an opportunity is presented in this study to test this inorganic compound for its ability to interfere hemoglobin-triggered oxidative and nitrative reactions. Such data are important in elucidating the roles of this simple anion in the mechanism of physiology and pathology related to heme proteins.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), enolase from *bakers yeast*, met-hemoglobin (Hb), sodium nitrite ($NaNO_2$), 2,4-dinitrophenylhydrazine (DNPH), rabbit polyclonal antibody against dinitrophenol (DNP) and 3-nitrotyrosine (3-NT) were purchased from Sigma. RPMI-1640 (for cell culture) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco BRL (Gaithersburg, MD, USA). All solvents and other reagents were of the highest purity commercially available.

2.2. Ferryl hemoglobin reduction by nitrite

Reactions between Hb and peroxides form the ferryl oxidation state of the protein, analogous to compounds I and II formed in the catalytic cycle of many peroxidase enzymes. This higher oxidation state of the protein is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules [4,5]. In order to investigate the definite effects of reducing agent on Hb-dependent oxidative reaction and eliminate potential side reaction, met-Hb-induced ferryl heme was usually selected [22–24]. Ferryl Hb was generated by addition of H_2O_2 to hemoglobin in a 1:1 M ratio at pH 7.4. After 15 min, catalase was added (10 U, final concentration) to remove excess H_2O_2, and then $NaNO_2$ was added. The final $NaNO_2$ concentrations were 50, 100, 200, 500 and 1000 μM for the experiments. The optical changes following addition of $NaNO_2$ were monitored and the time courses for reduction of ferryl Hb were fitted to a double exponential function [23,24]. The pseudo-first-order rate constants from these fits were then plotted as a function of $NaNO_2$ concentration. It should be noted that the stable ferryl Hb was partially formed from ferric Hb, due to the high reactivity of ferryl state [23–24] and the trace catalase in the commercially available Hb. The yield of the reaction of Hb with H_2O_2 (in a 1:1 M ratio) was approximately 25% and the partially ferryl form was used in the study. Moreover, the behavior of partially ferryl heme in Hb (an allosteric protein) would not be the same as fully populated systems nor ferryl myoglobin (a non-allosteric protein) [23–25].

2.3. Sulf hemoglobin measurement

The level of ferryl Hb was detected by its reaction with sodium sulfide (Na_2S) to form sulf Hb [25]. Typically 50 μM ferric Hb was used to react with 50 μM H_2O_2 followed by the reaction with increasing NO_2^- concentrations for a duration of 1 min. Then Na_2S (2 mM) was added to the reaction. The spectra of sulf Hb were measured, and the typical absorbance peak of sulf Hb was obtained at 620 nm [25].

2.4. Hemoglobin–H_2O_2-induced protein oxidation and nitration in the presence of nitrite

As the most abundant protein in plasma, BSA was usually used as the physiologically relevant substrate to investigate heme-induced protein oxidation and nitration [15,22]. Samples of BSA were treated with hemoglobin (20 μM)–H_2O_2 (0.5 mM), in the presence of different concentrations of NO_2^- in 50 mM phosphate buffer saline (PBS, pH 7.0) at 37 °C for 30 min. The final concentration of BSA was 0.5 mg/ml. Significant protein oxidative/nitrative modifications were observed in short time incubation when high concentrations of hemoglobin–H_2O_2–NO_2^- were used in many in vitro experiments [8,15]. These high concentrations were, therefore, chosen in our studies to conveniently compare the different effects of NO_2^-. The addition of $NaNO_2$ did not alter the pH of the reaction mixture significantly. The obtained reaction mixtures were used in latter assays. NO_2^- remaining in solution was measured by Griess method [26].

2.5. Western blotting analysis for protein oxidation and nitration

Oxidative damage of protein is accompanied by the formation of protein carbonyl groups that has been widely used as a marker of protein oxidation [20,21]. For detection of protein oxidation, the carbonyl groups in proteins were first derivatized with DNPH, resulting in the formation of DNP. The reaction was stopped with addition of neutralization solution, and then the DNPH-derivatized proteins were subsequently separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). For detection of protein tyrosine nitration, samples were directly mixed with loading buffer and subjected to SDS–PAGE. After electrophoresis, proteins were transferred to nitrocellulose membrane, and then immunoblotted with a rabbit polyclonal antibody against DNP or 3-NT. The antibody was detected using an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system. The optical density for Western blotting was performed and analyzed by Alpha Imager 2200 software.

2.6. Oxidative and nitrative modifications of enolase by hemoglobin–H_2O_2–NO_2^-

Since enolase was identified as the important and susceptive target for oxidative and nitrative modifications in many disease states [7,8,27], this widespread enzyme was chosen to reveal the relationship between protein nitrative/oxidative modifications and functional alteration. *Bakers yeast* enolase (0.1 mg/ml) was

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