RESEARCH COMMUNICATION

Reaction of peroxynitrite with oxyhaemoglobin: interference with photometrical determination of nitric oxide

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A frequently applied photometrical assay of NO is based on the reaction of NO with oxyhaemoglobin. This study shows that peroxynitrite induces spectral changes of oxyhaemoglobin identical with those elicited by NO. Like a variety of other agents,

peroxynitrite did not interfere with NO measurements using a Clark-type electrode, demonstrating that electrochemical detection has an advantage over the oxyhaemoglobin method for specific determination of NO.

INTRODUCTION

Cytotoxic effects of NO have been attributed to its fast reaction with superoxide, yielding peroxynitrite, which is stable in alkaline solutions but rapidly decomposes at physiological pH [1]. Decomposition of peroxynitrite involves generation of potentially cytotoxic radicals, which induce oxidation of DNA and lipid peroxidation [2]. It has been shown that cultured neurons are protected against glutamate toxicity by NO, but killed by peroxynitrite [3]. Taking into account these opposite biological effects, techniques for measurement of endogenous NO production should be specific and clearly distinguish between NO and peroxynitrite. Monitoring NO-induced spectral changes of oxyhaemoglobin (oxyHb) is claimed to be a specific assay for the determination of NO release from tissues or donor compounds [4,5], but here it is shown that peroxynitrite, either chemically synthesized or released from the sydnonimine SIN-1, mimics NO in the oxyHb assay.

EXPERIMENTAL

Materials

Peroxyntirite was synthesized and quantified photometrically (ϵ_{302} 1670 M⁻¹·cm⁻¹) as described in [6,7]. Alkaline stock solutions were stable at -70 °C for at least 2 months. NO solutions were prepared by dissolving NO gas in deoxygenated water [8]. 3-Morpholinosydnonimine (SIN-1), generously given by Cassella-Riedel (Frankfurt, Germany), was dissolved at pH 5.0 prior to use. Concentrations given for oxyHb refer to the monomer. Other chemicals and reagents, including bovine erythrocyte Cu,Zn-superoxide dismutase (SOD), were from Sigma Chemie (Deisenhofen, Germany).

Electrochemical determination of NO

Electrochemical determination of NO was performed with a Clark-type NO-sensitive electrode (ISO-NO, World Precision Instruments, Mauer, Germany) connected to an Apple Macintosh computer via an analogue-to-digital converter (MacLab, World Precision Instruments). Measurements were performed under constant stirring in 1.8 ml of a 50 mM triethanolamine/HCl buffer adjusted to pH 7.0 (NO, peroxy-

ntirite) or pH 8.0 (SIN-1) at ambient temperature. Unless otherwise indicated, the following solutions (mM) were added: 3 μ l of NO ($\sim$ 2), 0.9 μ l of peroxynitrite (75) and 18 μ l of SIN-1 (100).

OxyHb assay

Spectral changes of oxyHb were recorded at ambient temperature under constant stirring in the presence of 10–20 μ M oxyHb using a diode-array spectrophotometer (Hewlett-Packard, Vienna, Austria) as reported previously [9]. Incubation conditions and added stock solutions were as described for NO measurements. Peroxynitrite solutions were diluted 1:10 (v/v) with aq. distilled water or, for decomposition of peroxynitrite, with 0.1 M HCl. For quantitative analysis, loss of oxyHb was calculated from the difference in absorbances at 578 and 590 nm, using an ϵ value of 11.2 mM⁻¹·cm⁻¹ [10].

RESULTS

Figure 1 shows traces obtained with an NO-sensitive electrode on addition of NO, SIN-1 or peroxynitrite in the absence (Figure 1a) or presence (Figure 1b) of 5000 units of SOD/ml. When NO was added, maximal response of the electrode, corresponding to 1.9 μ M, was reached within 1 min. In accordance with previous reports on NO autoxidation (for a review, see [11]), decrease of the signal followed second-order kinetics with respect to NO. On addition of SIN-1 or peroxynitrite, NO concentrations were below the detection limit of about 20 nM. However, in the presence of high concentrations (5000 units/ml) of SOD, SIN-1 induced a pronounced response of the electrode corresponding to initial rates of about 0.4 μ M NO·min⁻¹. SOD did not affect the signals obtained with NO or peroxynitrite (Figure 1b).

Since oxidative cleavage of the A form of sydnonimines is thought to result in the production of superoxide anions along with NO [12], our data suggested that these species rapidly combine to peroxynitrite unless sufficient SOD is present for removal of superoxide. It has been reported, however, that SIN-1 exhibits NO-like activity in the oxyHb assay in the absence of SOD [4]. We have reinvestigated this issue and observed a loss of oxyHb at rates of 0.96 μ M·min⁻¹ in the presence of 1 mM SIN-1 at pH 8.0, and reaction rates were not significantly changed by 5000 units of SOD/ml (results not shown).

Figure 1 Electrochemical measurement of NO

Detection of NO was performed with a Clark-type NO-sensitive electrode at ambient temperature under constant stirring in the absence (a) or presence (b) of 5000 units of SOD/ml. Solutions of NO (3 μ l; 2 mM; —), SIN-1 (18 μ l; 100 mM; —) or peroxynitrite (0.9 μ l; 75 mM; ····) were added at zero time. Data are representative of those obtained in five similar experiments.

Figure 2 Spectral changes of oxyHb induced by NO and peroxynitrite

(a) Spectra were recorded in the presence of 12 μ M oxyHb at ambient temperature under constant stirring before (—) and 15 s after addition of solutions of NO (~ 10 μ M final concn.; ····) or peroxynitrite (37.5 μ M final concn.; —). (b) Difference spectra of oxyHb controls versus oxyHb after addition of NO (····) or peroxynitrite (—). Data are representative of those obtained in five similar experiments.

These results led us to speculate that peroxynitrite may react with oxyHb. We have synthesized, therefore, authentic peroxynitrite and found that it induced a shift of the Soret band and a decrease in absorbance of the β - and μ -bands of oxyHb identical with the spectral changes observed with NO (Figure 2a). Figure 2(b) shows the respective difference spectra. Reaction of oxyHb with peroxynitrite was complete within 15 s, and no spectral changes were observed when the alkaline stock solution had been diluted with 0.1 M HCl instead of water prior to the experiments (result not shown).

Figure 3 shows that loss of oxyHb increased linearly with increasing concentrations of peroxynitrite (slope = 0.15; $r = 0.9994$; $n = 4$), demonstrating that approx. 15% of added peroxynitrite was scavenged by oxyHb under these conditions.

Figure 3 Loss of oxyHb induced by increasing concentrations of peroxynitrite

Loss of oxyHb (~ 20 μ M) was measured over 15 s at ambient temperature in the presence of increasing concentrations of peroxynitrite and calculated as difference in the absorbances at 578 and 590 nm using an ϵ value of $11.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Data are means $\pm$ S.E.M. for four experiments.

The reaction with NO, however, was quantitative, as ~ 10 μ M NO consumed 10.7 ± 1.0 μ M oxyHb (mean $\pm$ S.E.M; $n = 4$).

DISCUSSION

Clark-type NO-sensitive electrodes appear to be highly selective for NO, since a wide variety of agents, including nitrite, nitrate, peroxynitrite, nitroxyl, superoxide and hydrogen peroxide, did not produce detectable signals (results not shown). The detection limit of the electrode was about 20 nM NO. Thus Clark-type NO electrodes are apparently less sensitive than the as-yet-not-commercially-available porphyrinic-based NO sensors [13], but allow a more sensitive detection of NO than the oxyHb method [4].

We used a Clark-type electrode to measure NO release from the sydnonimine SIN-1. On decomposition at alkaline pH, this drug concurrently generates NO and superoxide [12] and should therefore produce peroxynitrite rather than free NO. Consistent with this, we only observed NO release from SIN-1 when high amounts of SOD were present. Rates of NO release were 2.5-fold lower than SIN-1-induced loss of oxyHb, indicating that even concentrations of SOD as high as 5000 units/ml did not completely prevent peroxynitrite formation.

The discrepancy between lack of NO release from SIN-1 and its NO-like reaction with oxyHb may be explained by our findings that authentic peroxynitrite-induced spectral changes of oxyHb are indistinguishable from those elicited by NO. The reaction was unlikely to be mediated by residual nitrite present in the peroxynitrite preparations, because neutralization of the alkaline stock solutions prior to experiments resulted in a total loss of their activity in the oxyHb assay. The mechanisms underlying the effects of peroxynitrite are as yet elusive. It is possible that peroxynitrite directly oxidizes ferrous haem iron. Alternatively, the spectral changes we have observed could result from generation of NO during reaction of peroxynitrite with protein thiol groups [14]. However, since the reaction was complete within 15 s, its further characterization probably requires stopped-flow and rapid-scanning techniques as used recently for kinetic analyses of reactions of peroxynitrite with other haemoproteins, such as myeloperoxidase or catalase [15].

Peroxynitrite rapidly decomposes at physiological pH, owing to protonation to the labile peroxynitrous acid [1]. This may be the reason why only about 15% of added peroxynitrite was scavenged by oxyHb (see Figure 3). Similarly, low reaction yields were reported for various other peroxynitrite assays [16], and this

presumably reflects fast decomposition of exogenously added peroxynitrite. It should be considered, however, that scavenging of peroxynitrite may be much more effective when this species is continuously generated *in situ*.

The present results demonstrate that the oxyHb assay does not distinguish between NO and peroxynitrite. This method has been used, for example, to demonstrate release of NO during respiratory burst of human neutrophils [17], although it is evident from those results that all of the NO should have been converted into peroxynitrite by simultaneously generated superoxide. Our data strongly suggest that loss of oxyHb induced by the phorbol-ester-stimulated cells was due to release of peroxynitrite. Accordingly, the oxyHb assay appears to be of limited use when studying the involvement of NO in biological processes potentially encountered with concurrent release of superoxide.

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