Myeloperoxidase oxidation of sulfur-centered and benzoic acid hydroxyl radical scavengers

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Myeloperoxidase (MPO) oxidizes sulfur-centered and benzoate hydroxyl radical scavengers through formation of HOCl. Sulfur-centered hydroxyl radical scavengers compete with benzoate as antioxidants of HOCl. We conclude from these observations that competition experiments between benzoate and sulfur-centered hydroxyl radical scavengers are not sufficiently specific to infer participation of hydroxyl radicals in oxidative reactions mediated by neutrophils because of the unique action of MPO in affecting oxidation of the test radical scavengers.

Myeloperoxidase *Hydroxyl radical* *Neutrophil* *Hypochlorous acid* *Benzoate decarboxylation*

1. INTRODUCTION

In response to bacterial infections there is a sharp rise in the rate of O_2 consumption by neutrophils associated with activation of an NADPH:O_2 oxidoreductase on the surface of the cell [1]. Superoxide and H_2O_2 are products of the neutrophil oxidoreductase. Other more toxic O_2 metabolites may be secondary products of the oxidoreductase. Among the likely candidates is the hydroxyl radical. Its presence has been inferred based upon its reactivity as an oxidant of hydroxyl radical scavengers [2–7], and more recently by EPR techniques in which the radical has been detected as a nitroxide adduct [8,9]. Many of the hydroxyl radical scavengers employed are sulfur compounds. We demonstrate here that benzoic acid and sulfur-centered scavengers of hydroxyl radicals are unreliable in quantitating hydroxyl radical formation in the neutrophil. This is due to the formation of HOCl in the neutrophil and its oxidation of the radical scavengers.

2. MATERIALS AND METHODS

Superoxide dismutase (SOD), catalase, glucose oxidase, glucose, phorbol myristate acetate (PMA), *o*-dianisidine, dimethyl sulfoxide (DMSO), 2-keto-4-thiomethylbutyrate (KMB) and mannitol were obtained from Sigma, St. Louis, MO. Sodium azide was from Matheson Coleman and Bell, Norwood, OH. Reagent grade NaCl and H_2O_2 were obtained from J.T. Baker. Benzoic acid was obtained from Fischer Scientific, FairLawn, NJ. [*carboxyl*-^{14}C]Benzoic acid was obtained from ICN Pharmaceutical, Irvine, CA. HOCl was prepared by vacuum distillation of Chlorox (NaOCl) after adjusting the pH to 6.2 with dilute sulfuric acid; concentrations were determined using a molar absorptivity coefficient of 100 at 235 nm [10]. Myeloperoxidase (MPO) was prepared from human neutrophils essentially according to Merrill [11]. Enzyme activity was monitored by the *o*-dianisidine assay as in [12]. 1 mU corresponds to the oxidation of 1 nmol *o*-dianisidine per min. Neutrophils were isolated from whole blood and activated with PMA as described [13].

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The decarboxylation of benzoic acid was measured by the method of Winston and Cederbaum [14]. All results are expressed as the average of duplicate sets of assays.

3. RESULTS

The decarboxylation of labelled benzoic acid was measured and compared to that seen with omission of either MPO or NaCl, or with inclusion of either catalase or azide in cell-free reaction mixtures. In the complete system (table 1) the decarboxylation rate averaged 153 nmol per 30 min incubation at 37°C. Omission of MPO, NaCl, or addition of catalase to reaction mixtures, caused an approx. 3–5-fold diminution in the decarboxylation rate. Azide at 10^{-4} M, a known inhibitor of MPO [15], similarly inhibited decarboxylation of benzoate. HOCl could be substituted in place of MPO in affecting decarboxylation. Mannitol had no effect whereas 2 representative sulfur hydroxyl radical scavengers, DMSO and hypotaurine, which were added at concentrations normally employed in detecting hydroxyl radical formation, strongly inhibited decarboxylation (table 2).

Table 1

Myeloperoxidase-mediated decarboxylation of [*carboxyl*-^{14}C]benzoate

Reaction mixture	Decarboxylation rate[a] (nmol/30 min)	% control[b]
Complete system[c]	153 ± 0.1	100
+ 0.1 mM NaN_3	54 ± 5	35
+ catalase (1.8×10^3 U)	66 ± 3	43
+ boiled catalase	126 ± 1	82
− NaCl	35 ± 3	23
− MPO	52 ± 8	34

[a] Duplicate assays expressed ± SE

[b] Expressed as percent of decarboxylation rate measured in complete system

[c] Complete system includes 83 mM potassium phosphate, 10 mM potassium pyrophosphate, 5 mM glucose, 14 mU glucose oxidase, 100 mM NaCl, 10 mM benzoate (0.125 μCi), 330 mU MPO adjusted to pH 7.4; assays were initiated with addition of glucose oxidase to the reaction mixture (total volume 1 ml). Decarboxylation was measured as described in section 2

Table 2

Competition experiments between benzoate and hydroxyl radical scavengers as antioxidants of myeloperoxidase-generated HOCl

Reaction mixture	Benzoate decarboxylation rate[a] (nmol/30 min)	% control[b]
Expt I[c]		
complete system	150 ± 5	100
+ 30 mM mannitol	146 ± 11	97
+ 30 mM DMSO	36 ± 6	24
+ 5 mM hypotaurine	33 ± 6	22
Expt II[c]		
1 mM HOCl	276 ± 19	100
+ 30 mM mannitol	275 ± 4	100
+ 30 mM DMSO	26 ± 5	9
+ 5 mM hypotaurine	8 ± 2	3

[a] Duplicate assays expressed ± SE

[b] Expressed as percent of decarboxylation rate measured in the absence of competing hydroxyl radical scavengers

[c] In expt I the complete system was as noted in table 1; HOCl was substituted in place of MPO and glucose oxidase in reaction mixtures employed in expt II

Table 3

Effect of hydroxyl radical scavengers on benzoate decarboxylation in activated neutrophils

Experimental conditions	Decarboxylation rate[a] (nmol/30 min per 10^6 cells	% control[b]
Benzoate minus competing scavengers	71 ± 16	100
+ 30 mM mannitol	46 ± 8	64
+ 30 mM DMSO	37 ± 6	52
+ 5 mM hypotaurine	40 ± 4	56
+ 1 mM KMB	45 ± 27	63
+ SOD (150 U)	47 ± 29	66

[a] Duplicate assays expressed ± SE

[b] Expressed as percent of control rate measured in the absence of competing hydroxyl radical scavengers. Neutrophils were suspended at approx. 3.3 ± 10^5 cells · ml^{-1} in Hank's buffered saline solution, pH 7.4, and activated with addition of PMA (10 μg · ml^{-1}) to the reaction mixtures also containing 10 mM labelled benzoate (0.125 μCi) and the various scavengers as indicated. Decarboxylation rates were measured as described in section 2

Benzoic acid decarboxylation rates were also examined in activated neutrophil suspensions in the presence and absence of other hydroxyl radical scavengers. Rates ranged from approx. 65% for mannitol (30 mM), SOD (50 $\mu g \cdot ml^{-1}$) and KMB (1 mM) to 50% for DMSO (30 mM) and hypotaurine (5 mM) of control experiments with benzoic acid alone (table 3). In the absence of competing scavengers the absolute rate of benzoic acid decarboxylation was approx. 70 nmol/30 min per 10^6 activated cells.

4. DISCUSSION

The hydroxyl radical is a short-lived powerful oxidant. Its presence in reaction mixtures has been inferred indirectly by competition experiments taking advantage of its capacity to oxidize such chemical agents as benzoic acid, DMSO, KMB, dithiourea, methional, mannitol, *t*-butanol, or ethanol [2–7]. Selective iron chelators such as desferrioxamine and DTPA, catalase and SOD have also been added to reaction systems employing the indirect scavenging technique in search of hydroxyl radicals. This latter approach is predicated on the assumption that hydroxyl radicals may be formed by the Fenton reaction [5,16] in which Fe (or Cu), superoxide and H_2O_2 are all required to affect its production. Decreased oxidative damage to the hydroxyl radical scavenger with removal of either superoxide, H_2O_2, or trace metal (Fe or Cu) has been taken as supporting evidence for the presence of hydroxyl radicals.

Here, we have demonstrated that HOCl formed from MPO catalyzes oxidation of benzoate. This is evident by the marked diminution in the measured decarboxylation rate of benzoate seen with exclusion of either MPO or NaCl from the reaction mixtures, and by suppression of decarboxylation with inclusion of catalase in the final reaction mixtures (cf. table 1). Furthermore, from the model experiments with DMSO and hypotaurine (cf. table 2) we conclude that sulfur radical scavengers as a class are oxidative targets of HOCl as evident from the sparing effect of the sulfur radical scavengers on the decaboxylation rate of benzoate. These observations lead us to conclude that neither catalase nor sulfur radical scavengers are suitable probes in definitively establishing the presence of hydroxyl radical production in neutrophil preparations also rich in MPO. The suppression of benzoate decarboxylation with addition of KMB, hypotaurine or DMSO to activated neutrophils as shown in table 3, for example, may be either MPO or hydroxyl radical mediated, or possibly caused by a combination of both mechanisms.

Despite the ambiguities note above, the results of the mannitol and SOD scavenging experiments support the inference of hydroxyl radical production in the neutrophil. Clearly, mannitol is inert to HOCl as evident from the experimental results in table 2. The effect of SOD, on the other hand, suggests hydroxyl radicals may be forming in the activated neutrophil suspensions through a Fenton-type reaction in which superoxide is required for formation of the hydroxyl radical. These observations suggest that hydroxyl radical scavengers such as mannitol, *t*-butanol, ethanol, or SOD in ruling in or out Fenton-mediated hydroxyl radical production, may be more specific in building a case for hydroxyl radical formation in the neutrophil where potential interference from HOCl formation is likely. However, the effect of scavengers such as mannitol, *t*-butanol, and ethanol on the release and catalytic activity of MPO in the cell is unknown. Since MPO clearly oxidizes benzoate, variations in its catalytic activity or availability in the reaction mixtures affected by their presence in the reaction mixtures could be misinterpreted as evidence of different rates of hydroxyl radical production.

Klebanoff and Rosen [17] have presented evidence that MPO oxidizes KMB to form ethylene in phagocytizing neutrophils through production of HOCl. Weiss et al. [3] using KMB and Sagone et al. [2] using benzoate as hydroxyl radical scavengers observed marked inhibition of ethylene production and $^{14}CO_2$ evolution, respectively, with azide added to cell suspensions. The effect of azide strongly suggests MPO may have been involved in some of the oxidative reactions measured by both groups. Albrich et al. [18] have demonstrated through model experiments that HOCl is a powerful oxidant of sulfhydryl groups and that their reactivity with HOCl roughly parallels their nucleophilic reactivity. All of the above observations suggest that inferences regarding the participation of hydroxyl radicals in neutrophil suspensions should be evaluated cautiously with respect to interfering oxidative reactions mediated by MPO.

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