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A NEW METHOD FOR THE DETECTION OF HYDROXYL RADICAL PRODUCTION BY PHAGOCYTIC CELLS

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Summary

Benzoic acid, a specific scavenger of hydroxyl radical ($OH^{\cdot}$) is known to be oxidized as the result of a reaction with $OH^{\cdot}$. We have determined that the decarboxylation of benzoic acid can be used to detect $OH^{\cdot}$ generated in cell-free systems and human granulocytes.

Benzoic acid is oxidized by the xanthine-xanthine oxidase enzyme system. This system is known to generate O_2^-, H_2O_2 and $OH^{\cdot}$. This oxidation is inhibited by superoxide dismutase, catalase and mannitol. Therefore, the oxidation of benzoic acid occurs by a mechanism similar to that reported for the oxidation of methional to ethylene and involves $OH^{\cdot}$.

Resting granulocytes do not oxidize benzoic acid. However, marked oxidation of this substrate occurs during the phagocytosis of opsonized zymosan particles, indicating the production of $OH^{\cdot}$ by these cells. The reaction can be inhibited by superoxide dismutase, catalase, azide and mannitol. Therefore, the production of $OH^{\cdot}$ in the cell may be similar to that observed in the cell-free system.

The granulocytes of a patient with known chronic granulomatous disease did not oxidize benzoic acid, indicating a defect in the generation of $OH^{\cdot}$ by these cells.

Introduction

Phagocytic cells generate several highly reactive oxygen species which are known to be important in their function. These include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and possibly singlet oxygen [1—10].

Direct evidence that phagocytic cells generate $OH^{\cdot}$ has been reported by several laboratories [3—5,10]. Weiss et al. [4,5] and Tauber and Babior [3] demonstrated that both methional and 2-keto-4-methylthiobutyric acid were oxidized to ethylene by both human granulocytes and monocytes [3—5]. These reactions appear to involve $OH^{\cdot}$ radicals. Similar results have now been reported with rat granulocytes and alveolar and peritoneal macrophages [10]. However, some difficulties have been reported with these assays. Weiss reported that methional is readily autooxidizable, making it somewhat difficult to use [4]. Using 2-keto-4-methylthiobutyric acid he reported that $OH^{\cdot}$ appears to be dependent on both O_2^- and H_2O_2 production by granulocytes (Haber-Weiss reaction). Tauber and Babior reported that $OH^{\cdot}$ generation appears dependent only on O_2^- and appears not to require H_2O_2 [3]. The reasons for these differences are not clear. Babior postulated, however, that methional might undergo spontaneous oxidation to an organic hydroperoxide which could then react directly with $OH^{\cdot}$ [1,2]. In contrast, 2-keto-4-methylthiobutyric acid may require interaction with H_2O_2 before it can react with $OH^{\cdot}$, resulting in an apparent requirement for both O_2^- and H_2O_2 in the production of $OH^{\cdot}$ by the cells.

Many studies addressed to the detection of $OH^{\cdot}$ generation in cell-free systems and by cells have utilized benzoate as a scavenger [1—8,11,12] since it is specific for $OH^{\cdot}$. In this regard, benzoic acid is known to be decarboxylated to CO_2 during interaction with $OH^{\cdot}$, a reaction which has been shown to be specific [13,14]. The oxidation of benzoic acid, therefore, has been used to quantitate production of $OH^{\cdot}$ in irradiated solutions [13] and in the ozonation of water [14]. This raised the possibility that [^{14}C]benzoate could be used as a sensitive assay to quantitate the generation of $OH^{\cdot}$ by phagocytic cells. In the experiments reported here, we demonstrate that granulocytes oxidize the carboxyl group of benzoic acid to CO_2 during the phagocytosis of zymosan particles. This observation provides further evidence that granulocytes generate $OH^{\cdot}$. Using this assay we have investigated further the mechanism of $OH^{\cdot}$ generation by phagocytic cells.

Materials and Methods

Methods

Cell preparation. Granulocytes were isolated by Dextran sedimentation from normal volunteers and from a patient known to have chronic granulomatous disease [15]. The cells were purified further by Ficoll-Hypaque gradient centrifugation to remove contaminating mononuclear cells as previously reported [15]. The final preparation contained greater than 90% granulocytes, less than 10% erythrocytes, and less than 1% mononuclear cells.

Metabolism of benzoate by granulocytes. The production of $^{14}CO_2$ from [^{14}C]benzoic acid by granulocyte suspensions incubated at 37° C was measured continuously using the ionization chamber-electrometer method as described previously [9]. Twenty million granulocytes were resuspended in a 25 ml triple-arm distilling flask. The suspension was placed into 4 ml of Earle's balanced salt solution with 50 mg/100 ml of glucose containing 185 kBq of

[^{14}C]benzoic acid in the carboxyl ^{14}C form. The final concentration of benzoic acid was 0.025 mM.

The inlet arm of the metabolic flask was connected to a gas cylinder containing compressed air with 5% CO_2. The outlet arm of the flask was connected to a 275 ml Cary-Tolbert ionization chamber and a Cary model 401 vibrating reed electrometer (Cary Instruments). The third arm of the flask was covered with a rubber stopper. A duplicate system was used so that $^{14}CO_2$ derived from [^{14}C]benzoic acid could be measured simultaneously from both control and experimental flasks. The incubation flasks were stirred continuously using a Magne-4 Magnetic Stirrer, Model 3820-1 (Cole-Palmer Instrument Co.) with spin bars in the flasks. After baseline CO_2 production was established, opsonized zymosan particles were added in 0.4 ml of normal saline ($4 \cdot 10^8$ particles/ml). In studies in which the effects of enzymes or scavengers were determined, the active or heat denatured enzymes were added in 0.1 ml of buffer before the addition of the zymosan particles. An equal volume of buffer was added to the controls. CO_2 production was calculated from the millivolt reading and expressed as nmol of CO_2 produced per h per 10^7 cells. The peak value achieved after the addition of zymosan was used to compare values between control and experimental flasks.

Cells were counted electronically using a model FN Coulter Counter (Coulter Electronics Inc.).

Experiments with cell-free systems. The effect of xanthine ($1 \cdot 10^{-4}$ M) and xanthine oxidase (0.0075 unit/ml) on the oxidation of [^{14}C]benzoic acid in a cell-free system was studied in order to determine the specificity of benzoate oxidation. This enzyme system has been documented to generate several reactive oxygen species [12]. 185 kBq of [^{14}C]benzoic acid were resuspended in 4 ml of Dulbecco's phosphate buffered saline (GIBCO) and the acid's oxidation was studied continuously at 37°C using the ionization-chamber electrometer system as described above for experiments involving cells, except that the medium was gassed with air rather than air/CO_2. The effect of scavengers was also evaluated. The scavengers, including superoxide dismutase, catalase and mannitol, were added to the solution before the addition of the enzyme system.

Materials

Superoxide dismutase (3000 U/mg) was purchased from Truett Labs, Dallas, TX; xanthine, xanthine-oxidase (Sigma Grade 1, 0.75 U/mg protein), catalase (beef liver-thymol free 13 000 U/mg), sodium azide, Bovine serum albumin, and zymosan were purchased from Sigma. Zymosan was opsonized in freshly filtered human AB sera as described by Webb [6]. Mannitol was purchased from Merck, Sharp, and Dohme. [^{14}C]Benzoic acid was purchased from Amersham Corporation, Arlington Heights, IL. (Spec. act. 2109 GBq/mol) and was dissolved in 5 ml of sterile 0.9% saline. For the experiments, 185 kBq of [^{14}C]benzoic acid were added to each cell suspension. In some cases, small quantities of volatile radioactive materials were found in the radioactive materials and were removed by gassing before the addition of other reagents.

Statystical analysis

Data were analyzed according to the *t*-test for independent samples [6].

Results

Effect of enzyme system xanthine-xanthine oxidase on the oxidation of benzoate (cell-free system)

The enzyme system xanthine-xanthine oxidase is known to generate several reactive oxygen compounds which include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$) [12]. For this reason we determined whether benzoate could be oxidized by this enzyme system in order to identify the specificity of decarboxylation of benzoate by $OH^\cdot$.

Beauchamp and Fridovich demonstrated that methional is oxidized to ethylene by this enzyme system [12]. The production of $OH^\cdot$ in this system is dependent on the interaction of O_2^- with H_2O_2 (Haber-Weiss reaction). As indicated in Table I, benzoate oxidation to $^{14}CO_2$ occurs during interaction with xanthine-xanthine oxidase system. Similar to the oxidation of methional, this reaction is dependent on both O_2^- and H_2O_2 as indicated by the inhibitory effects of both SOD and catalase. In addition, the oxidation of benzoate could be inhibited by mannitol, a known scavenger of $OH^\cdot$. 0.1 mM EDTA added to the incubation augmented benzoate oxidation. The etiology of this is not clear but may be related to chelation of small amounts of heavy metal cations such as iron by the EDTA, which may inhibit the activity of the enzyme or alternately enhance the Haber-Weiss reaction [12].

Oxidation of [^{14}C]benzoate by granulocytes

The oxidation of [^{14}C]benzoate by granulocyte suspensions is illustrated in Fig. 1. As indicated, resting granulocyte did not oxidize this substrate. In contrast, the addition of opsonized zymosan particles to the suspensions was associated with an immediate increase in the oxidation of [^{14}C]benzoate which

TABLE I

OXIDATION OF [*carboxyl*-^{14}C]BENZOIC ACID IN CELL-FREE SYSTEM

The complete system consisted of $1 \cdot 10^{-4}$ M xanthine, 0.0075 unit/ml xanthine and 5 μCi of [*carboxyl*-^{14}C]benzoic acid (0.025 mM). Additions and deletions were made to the complete system. These included superoxide dismutase (10 μg/ml), catalase (25 μg/ml), mannitol 40 mM, autoclaved superoxide dismutase (10 μg/ml), 0.1 mM EDTA, and autoclaved catalase (25 μg/ml) in Dulbecco's phosphate buffered saline.

Components	$^{14}CO_2$ production *
Complete system	0.25
— Xanthine	0
— Xanthine oxidase	0
+ Superoxide dismutase	0.02
+ Catalase	0.01
+ Mannitol	0.02
+ Autoclaved superoxide dismutase	0.23
+ Autoclaved catalase	0.27
+ EDTA	0.59

* Peak rate of $^{14}CO_2$ produced per h (nmols).

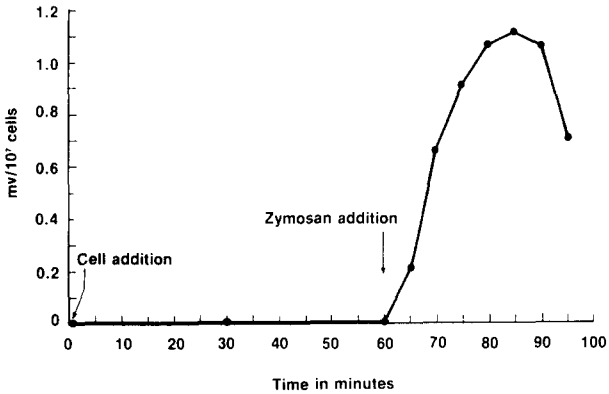

Fig. 1. The oxidation of [^{14}C]benzoate by granulocyte suspensions. The curves represent a continuous measurement of $^{14}CO_2$ production by granulocyte suspensions in buffer using the ionization chamber-electrometer system. The curves were drawn from the data points of a single experiment which is representative of experiments performed. Values are in mV produced per 10^7 cells. The addition of opsonized zymosan to the suspension is indicated by the arrow.

peaked within 20 to 30 min and then gradually decreased toward baseline. The mean peak value for 37 experiments was 0.14 ± 0.04 (S.D.) nmol/10^7 cells per h with a range of 0.075 to 0.26. The addition of zymosan alone without cells did not stimulate the oxidation of benzoate. Benzoate alone in the concentrations used in our experiments does not impair hexose monophosphate shunt activity, formate oxidation or the generation of chemiluminescence by the polymorphonuclear leukocyte system (Ref. 17 and unpublished observations).

Effect of scavengers on the oxidation of benzoate

Once we observed that suspensions of granulocytes oxidized [^{14}C]benzoate, we studied the effect of several agents which are known scavengers of the reactive oxygen compounds generated by granulocytes. These included superoxide dismutase, catalase and mannitol.

Superoxide dismutase (10 μg/ml) impaired the oxidation of [^{14}C]benzoate 39% (Fig. 2 and Table II). The value observed for suspensions supplemented with superoxide dismutase was 61% of the control value. Heat-denatured superoxide dismutase had no effect, indicating a requirement for active enzyme. More significant impairment of oxidation was observed as the concentration of superoxide dismutase was increased to 200 μg/ml. The inhibition with this amount of the enzyme was 68%. However, when the enzyme was heat inactivated, 55% inhibition was still observed, indicating that this high concentration of superoxide dismutase was associated with a non-specific scavenging effect. Benzoate oxidation was also studied in suspensions supplemented with catalase (Table II). This enzyme also impaired the oxidation of [^{14}C]benzoate by granulocytes during the phagocytosis of zymosan particles. Inactivation of the enzyme eliminated its inhibitory effect.

Bovine serum albumin in a similar concentration to superoxide dismutase and catalase did not impair benzoate oxidation. Thus, the effect of these

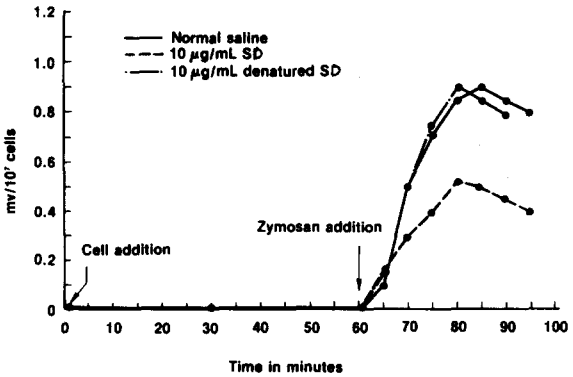

Fig. 2. Effect of superoxide dismutase (S.D.) on the oxidation of [^{14}C]benzoate. Superoxide dismutase (10 μg/ml) was added to one suspension (- - - - - -). The time and addition of opsonized zymosan to both suspensions are indicated by the arrow. Heat inactivated dismutase was added to some suspensions as controls (· — · — ·). Similar experiments were performed with catalase, mannitol or azide. The results with these agents are summarized in Table II.

enzymes does not appear to be nonspecific. As expected, mannitol (40 mM), a known hydroxyl radical scavenger, impaired the oxidation of benzoate (Table II). Azide is a compound which can affect granulocyte metabolism in several diverse ways. As indicated in Table II, a $1 \cdot 10^{-4}$ M concentration of this agent almost completely inhibited the oxidation of benzoate.

Similar experiments were done with the granulocytes of a patient with chronic granulomatous disease. These granulocytes generated barely detectable amounts of $^{14}CO_2$ during the phagocytosis of zymosan particles, indicating that these cells have a marked impairment in the ability to generate $OH^{\cdot}$.

TABLE II

OXIDATION OF BENZOATE BY GRANULOCYTES INCUBATED WITH VARIOUS AGENTS

The oxidation of benzoate by granulocytes incubated with each agent was compared to the corresponding control in three paired experiments. The kinetics in the presence of each agent were similar to those of the control. An example for superoxide dismutase is given in Fig. 2. All values are significantly different than corresponding control ($P < 0.05$), except for those done with autoclaved superoxide dismutase, catalase and albumin.

Scavenger	Benzoate oxidation (% of control ± S.D.)
None	100
+ Superoxide dismutase, 10 μg/ml	61 ± 2.8
+ Autoclaved superoxide dismutase, 10 μg/ml	100
+ Superoxide dismutase, 200 μg/ml	32 ± 7.8
+ Autoclaved superoxide dismutase, 200 μg/ml	45.0 ± 1.1
+ Catalase, 25 μg/ml	67.3 ± 1.5
+ Autoclaved catalase	94.1 ± 4.7
+ Mannitol, 40 mM	42.0 ± 5.6
+ Azide, $1 \cdot 10^{-4}$ M	10.3 ± 0.5
+ Albumin, 25 μg/ml	96.0 ± 8.0

Discussion

Benzoic acid is known to be decarboxylated to CO_2 in the presence of hydroxyl radicals [13]. This specific chemical reaction has been used to quantitate the generation of $OH^{\cdot}$ in irradiated solutions [13] and to detect the generation of $OH^{\cdot}$ from ozone [14]. This raised the hypothesis that the oxidation of benzoate might be used to detect the generation of $OH^{\cdot}$ by phagocytic cells. Experiments with a cell-free system using xanthine-xanthine oxidase indicated that benzoate is oxidized to CO_2 by a reaction similar to that reported for methional and thereafter appears to be related to $OH^{\cdot}$ [12]. Our experiments with granulocytes indicate that these cells oxidize benzoate during the phagocytosis of opsonized zymosan particles.

The pattern of the oxidation of benzoate by granulocytes is similar to that which we have previously reported for oxidation of 1-[^{14}C]glucose and [^{14}C]-formate [9] indicating that all of these biochemical events are closely related. The observation that the oxidation of benzoate could be inhibited by mannitol, a known $OH^{\cdot}$ scavenger, indicates that the oxidation of benzoate is related to $OH^{\cdot}$. The concentration of mannitol used in our experiments does not significantly impair the stimulation of the hexose monophosphate pathway, or generation of superoxide, H_2O_2, or chemiluminscence, indicating that the effect of mannitol is specific (unpublished observations). Thus, our experiments suggest that granulocytes generate $OH^{\cdot}$, as previously suggested by others using different assays [3,5,10]. Our results do not exclude, however, the possibility that radicals other than $OH^{\cdot}$, such as organic peroxides, contribute to the oxidation of benzoic acid. This possibility requires further study. The granulocytes of a patient with chronic granulomatous disease did not significantly oxidize benzoate during the phagocytosis of zymosan, confirming that these cells do not generate $OH^{\cdot}$.

Our experiments indicate that the generation of $OH^{\cdot}$ by the cells may be partially dependent on the generation of both superoxide and H_2O_2 as indicated by the inhibitory effect of both superoxide dismutase and catalase. This is similar to the observations of Weiss et al. [5] using a different assay. However, although Tauber and Babior demonstrated a role for O_2^-, they reported that there was a non-specific inhibitory effect of catalase [3]. Our experiments indicate that catalase does appear to have a definite, specific protective effect, indicating a role of H_2O_2 in the generation of $OH^{\cdot}$. However, the protection was small. In this regard we also observed that a nonspecific protective effect of superoxide dismutase occurred when a high concentration of the enzyme was used. This probably reflects the high reactivity of $OH^{\cdot}$ for protein in general and must be considered when these scavengers are used to identify the role of O_2^- and H_2O_2 in the functional capacity of the cell.

The concentrations of catalase and lower concentration of superoxide dismutase used in our experiments do not impair the oxidation of benzoic acid during irradiation (unpublished observations). Thus, the effects of the enzymes in our experiments do not appear to be related to a nonspecific one.

Whether the supression of $OH^{\cdot}$ by superoxide dismutase and catalase indicates a requirement for H_2O_2 and O_2^- to intereact to generate $OH^{\cdot}$ (Haber-Weiss reaction) [11] or whether these agents are acting independently is not clear and requires further study.

Benzoate oxidation by granulocytes was almost completely inhibited by azide, suggesting a requirement of heme, a heme-containing enzyme such as myeloperoxidase or possibly singlet oxygen in the reaction. Our experiments appear to exclude a role of catalase, since this enzyme impaired the oxidation of benzoate rather than stimulating it and did not promote its oxidation by the xanthine-xanthine oxidase system. In this regard, the major mechanisms for oxidation of [^{14}C]formate by phagocytic cells appear to differ significantly from that of benzoate and appear to be dependent primarily upon H_2O_2 and catalase [9,18,19].

In summary, we report here a new simple sensitive assay for the detection of $OH^{\cdot}$ generation by phagocytic cells. Using this assay we were able to show that the generation of $OH^{\cdot}$ by granulocytes is partially dependent on O_2^-, H_2O_2 and is inhibited by azide. The assay appears to afford some advantages over the assays which have been previously reported. We have noted no difficulty with spontaneous oxidation of this compound and benzoate does not need to be converted into an intermediate, as may be the case with methional, to react with $OH^{\cdot}$. Further, the concentration of [^{14}C]benzoate used in our experiments does not appear to alter the biochemical response of granulocytes to zymosan particles as measured by other assays, indicating that our $OH^{\cdot}$ assay does not alter this complex system. The effect of methional and 2-keto-4-methylbutyric acid on hexose monophosphate activity, H_2O_2 and O_2^- production of granulocytes, either resting or during phagocytosis, has not as yet been reported.

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