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Metabolism of benzoic acid by stimulated polymorphonuclear cells*

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Polymorphonuclear cells (PMNs) are capable of generating reactive oxygen species (ROS) such as superoxide radical anion and hydroxyl radicals when stimulated [1–5]. These transient species are highly reactive and can interact both with macromolecular constituents of the cell and xenobiotic materials.

When stimulated by opsonized zymosan particles, human PMNs respond with a burst of oxidative activity [2]. We have shown previously that stimulated PMNs are capable of decarboxylating benzoate, although the exact nature of the attacking species has been debated [3, 4]. We have also shown that the decarboxylation reaction can be inhibited by specific radical scavengers such as mannitol and dimethyl sulfoxide (DMSO) [3, 6]. Others have shown, using both ionizing radiation and nonbiologic chemical reactions, that the aromatic hydroxylation of benzoate appears to be hydroxyl-specific [7–9].

In this paper, we report the results of experiments testing the benzoate hydroxylation ability of stimulated PMNs *in vitro*. Human blood granulocytes were obtained from the venous blood of human volunteers [10, 11]. Approximately 40 ml of blood was drawn by syringe from a peripheral arm vein into a mixture of EDTA and dextran solution. After mixing, the erythrocytes were allowed to sediment under normal gravity for 55 min. The upper plasma layer containing PMNs was carefully removed, mixed with cold Seligman's balanced salt solution (SBSS), and layered over Ficoll–Hypaque solution in 50 ml plastic centrifuge tubes. After centrifugation, the solutions were aspirated from the PMN pellet. The cells were subjected to shock lysis with distilled water to remove residual erythrocytes [10, 11]. The cells were suspended in Dulbecco's phosphate-buffered saline with 5 mM glucose, to a final concentration of 20×10^6 cells/ml [11]. Zymosan was opsonized by incubation with autologous serum for 30 min in a 37° bath [10, 11]. The zymosan particles were recovered by centrifugation and resuspended in the same medium used for the cells. An aliquot of the cell suspension (1 ml) was placed in a plastic tube containing a small magnetic stirring bar. The cells were preincubated for 10 min with 20 mM benozate and either mannitol (20 mM), DMSO (30 mM), azide (0.1 mM), superoxide dismutase (SOD) (15 μg/ml) (Sigma 2500 units/mg) or catalase (20 μg/ml) (Sigma 20,000 units/mg). Zymosan was then added to initiate the reaction (final concentration 5 mg/20×10^6 cells), and the cells were incubated at 37° for 60 min with constant stirring. Control incubations of cells were performed in each case with benzoate and zymosan alone, to establish full activity levels, and with benzoate alone, to correct for any activity in unstimulated cells. In the latter, no activity was ever observed. Incubations were halted by immersion of the tubes in an ice-water bath for 10 min. The material was then stored at −20° until analysis could be performed.

The sample were analyzed by high performance liquid chromatography (HPLC). A 400-μl aliquot of the incubate in a small test tube was acidified with phosphoric acid and spiked with *o*-methoxybenzoic acid as internal standard. This was extracted with 4 ml of diethyl ether. After transferring the ether to a second tube, 20 μl of 1 N sodium hydroxide was added, and the solvent evaporated under nitrogen. The addition of base was to prevent sublimation of benzoic acid during the evaporation process. The sample was then reconstituted with the chromatographic mobile phase and injected onto the column. The chromatograph used was an Altex MP 322 dual pump system operated in isocratic mode. The mobile phase was a mixture of isopropanol and 5% aqueous acetic acid containing 2.0 g/l ammonium acetate (5:95). The flow rate was 1.0 ml/min using a Beckman Ultrasphere C-18 4.6 × 250 mm column. Detection was by ultraviolet at 240 nm, using a Beckman model 165 variable wavelength detector (Beckman Instruments, Irvine, CA).

As seen in Fig. 1, PMNs incubated with benzoate alone showed no evidence of benzoate hydroxylation ability. However, when cells were stimulated with opsonized zymosan in the presence of benzoate, analysis of the 60 min supernatant fraction showed the presence of hydroxylated products. All three possible monohydroxybenzoate isomers could be detected. Typically, the final concentration of ortho-, meta- and para-hydroxybenzoate (HBA) totalled 5 μM, corresponding to production levels of 3.8 nmoles/

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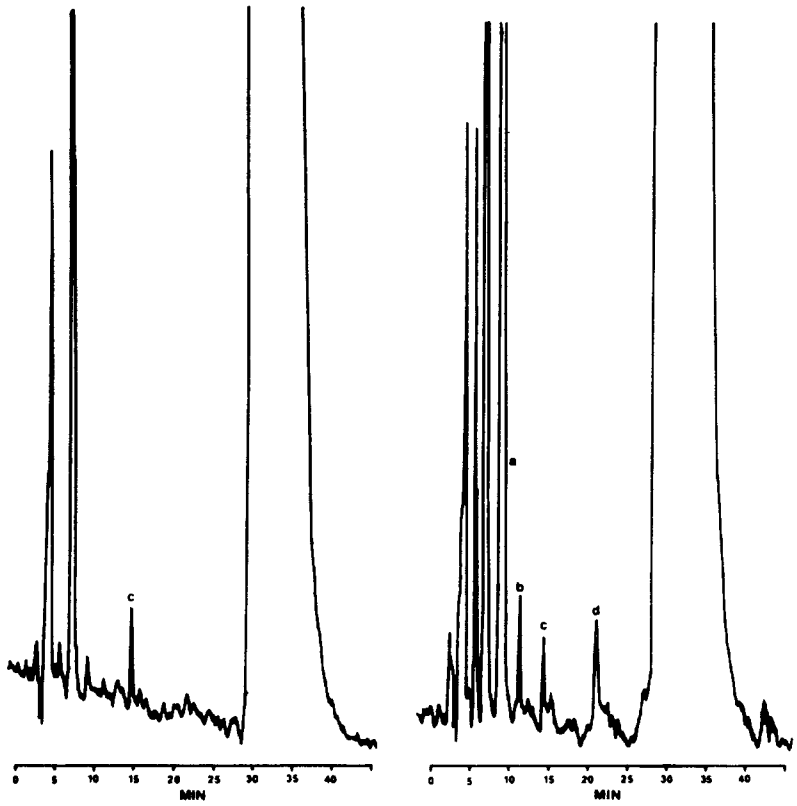

Fig. 1. Liquid chromatograms of supernatant from PMNs incubated with benzoate. Left: PMNs only. Right: PMNs plus opsonized zymosan. Key: (a) para-HBA; (b) meta-HBA; (c) standard; and (d) ortho-HBA. Benzoate is the off scale peak at the right in each scan. Actual concentrations of each compound were calculated by the peak area ratio method using standard curves to correct for the different molar extinction coefficients.

10^7 cells/hr. The product ratios found were not those that would be expected from random attack, i.e. ortho : meta : para = 2 : 2 : 1, but were closer to 1 : 4 : 1. Pre-incubation with 20 mM mannitol reduced the production of hydroxylated benzoate by an average of 35% versus control. DMSO, in a concentration of 30 mM, caused a 40% reduction in hydroxylation. Azide, at a 0.1 mM concentration, reduced hydroxylation by about 60%. The addition of SOD (15 μg/ml) caused a reduction of about 35% versus control. The effect of catalase (20 μg/ml) was insignificant. Figure 2 shows the final results, expressed as percent of hydroxylated material formed compared to control incubations of PMNs with benzoate and zymosan.

The results of this study offer further evidence that the species responsible for at least some of the oxidative activity in stimulated PMNs is the hydroxyl radical [2–5]. Others have shown that benzoate hydroxylation is hydroxyl-specific [7–9]. Hydroxyl can be generated in aqueous solution by the interaction of superoxide radical anion and hydrogen peroxide, either by a Haber–Weiss type of reaction or via a Fenton-type reaction, in which a metal ion acts as the electron-transfer agent [1, 2, 5, 7]. Mannitol and DMSO, both known radical scavengers, can compete with benzoate for free radicals. SOD destroys superoxide by catalyzing its conversion to hydrogen peroxide and molecular oxygen, and hydrogen peroxide can be broken down by catalase. Whatever the route of formation, the presence of ring-hydroxylated products offers direct evidence for the existence of free hydroxyl in the stimulated cell.

The ability of SOD to suppress the reaction suggests that O_2^- is required for hydroxyl production, although whether it is produced via a Haber–Weiss or Fenton pathway is not clear. It is also not obvious why catalase is unable to suppress the reaction. It may be a matter of poor penetration of the cell membrane, or rapid destruction by cellular proteases.

The levels of hydroxylated products formed are comparable to the amount of decarboxylation that occurs in the same system [3, 4]. The product ratios are not what would be expected from a random attack on the benzoate ring by hydroxyl. However, unpublished data obtained in this laboratory indicate that salicylate undergoes decarboxylation more readily than benzoate. It may be that salicylate is further reacting at the formation site by decarboxylation, reducing its final concentration. The expected nonvolatile products of such a reaction, phenol and/or resorcinols, have not been detected, so such a statement remains speculative. The fact that the para isomer ratio is also lower than expected does suggest that the decarboxylation transition state is stabilized by ortho para-ring substitution, facilitating the loss of carbon dioxide.

Caution must be exercised in directly comparing results obtained from differing systems. Our results suggest, however, that drugs may be metabolized at a site of inflammation by granulocytes. This could result in compounds with either increased or decreased biological activity depending on the nature of the parent drug. In any event, our experiments show that the PMN, when stimulated, can chemically modify xenobiotic materials by ring hydroxylation. The production of a pharmacologically active material within a stimulated leukocyte *in vivo* could be of great importance.

In summary, stimulated granulocytes are known to release several reactive oxygen species. We have demon-

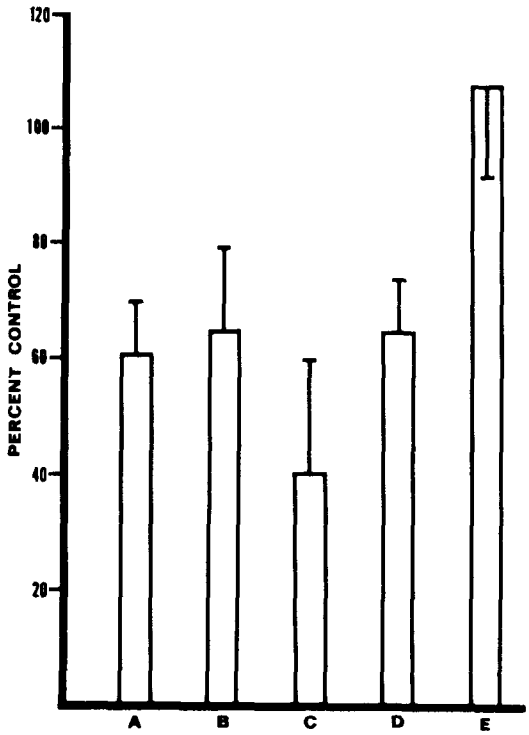

Fig. 2. Inhibition of benzoate hydroxylation expressed as percent products formed versus paired control. Key: (A) DMSO, (B) mannitol; (C) azide; (D) SOD; and (E) catalase.

strated that the hydroxyl radical produced by these cells oxidizes benzoic acid. This observation predicted that the $OH^{\cdot}$ produced by granulocytes would also cause ring hydroxylation of this drug. This possibility was tested in our current experiments. We were able to demonstrate the production of three monohydroxybenzoate isomers—ortho-, meta- and para-hydroxybenzoate—after solutions of benzoate were incubated with stimulated granulocytes. These data provide additional evidence that human PMNs generate $OH^{\cdot}$. Of importance is that our observations suggest that stimulated PMNs may chemically modify xenobiotic materials by ring hydroxylation and thereby their activity at a site of inflammation *in vivo*.

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Chlorphentermine-induced alterations in the response of human lymphocytes to mitogens

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In recent years numerous studies have shown that the administration of certain cationic amphiphilic drugs (CADs) to animals, and in some instances humans, results in the induction of a lysosomally-derived phospholipidosis in many tissues of the body [1–3]. The hallmark feature of the disorder is the development of phospholipid-rich lamellar inclusions in association with lysosomes in the cell [4]. Of the CADs reported to induce this disorder, chlorphentermine (CP) has been studied the most extensively [1, 5, 6]. One cell type that has been reported to be quite sensitive to the induction of phospholipidosis by CP is the lymphocyte of peripheral blood [6]. The administration of a single dose of this drug to rats results in the appearance of lamellar inclusions in the cell. Other CADs including citalopram [7] and a number of tricyclic antidepressants [8] are capable of inducing the disorder in lymphocytes present in peripheral blood or lymph nodes. At present, we are aware of no studies investigating the consequences of exposure to CP, or any other CAD reported to induce phospholipidosis in this cell, on lymphocyte function. We have initiated studies to address this problem, and in this communication report on the effects of CP exposure *in vitro* on mitogen-induced blastogenesis in human peripheral blood lymphocytes.

Materials and methods

Peripheral blood (30 ml) was drawn from normal, healthy males by venipuncture into heparinized Vacutainer blood collection tubes (Becton Dickinson, Rutherford, NJ). All subjects were volunteers who signed a consent form approved by the West Virginia University Institutional Review Board for the Protection of Human Subjects.

All cultures were carried out in RPMI 1640 medium (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 5×10^{-5} M mercaptoethanol, 10 mg pyruvate/100 ml, 2 μg asparagine/100 ml (Sigma, St. Louis, MO), 100 units penicillin/100 ml (Squibb, Princeton, NJ), 100 μg