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HYDROXYLATION OF SALICYLATE BY ACTIVATED NEUTROPHILS

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Abstract—Salicylates are metabolized *in vivo* to hydroxylated compounds, including 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid (gentisic acid). The present study hypothesized that activated neutrophils represent one pathway for salicylate hydroxylation. Human neutrophils were incubated in medium containing 10 mM salicylate and stimulated with phorbol myristate acetate (PMA) for 1 hr. The cell-free supernatant fractions were analyzed by HPLC. Neutrophils (1×10^6 cells) produced 55 ± 11 ng of gentisic acid. Neutrophils also produced smaller quantities of 2,3-dihydroxybenzoic acid. Antioxidant inhibitor experiments indicated that superoxide dismutase (SOD), heme protein inhibitors, and glutathione blocked gentisic acid formation, whereas catalase, mannitol, and deferoxamine failed to inhibit. Experiments with the reagent hypochlorous acid (HOCl) and the model myeloperoxidase (MPO) enzyme system did not support a role for the MPO pathway in gentisic acid formation. These findings demonstrate that activated neutrophils can hydroxylate salicylate by an unknown pathway. This pathway may contribute to the increased recovery of hydroxylated salicylates in patients with inflammatory disorders.

There is increasing evidence that the salicylate family of compounds can be metabolized by activated inflammatory cells [1–10]. For example, Dull *et al.* [1] demonstrated that 5-aminosalicylic acid (5-ASA‖) is metabolized to salicylate and gentisate (2,5-dihydroxybenzoate) by activated mononuclear cells and neutrophils. Williams and Hallett [2] showed that 5-ASA is metabolized in a reaction in which 5-ASA decreases the luminol-dependent chemiluminescence of neutrophils. Our laboratory has shown previously that activated neutrophils can decarboxylate benzoate [4–6] and salicylate [7], and that hydroxylated benzoate metabolites can be detected by HPLC [8]. All of these studies demonstrate that salicylate compounds are metabolized by reactions involving reactive oxygen species. Although the exact biochemical reactions in the intact cell remain uncertain, many of the reactions have also been studied using oxidants in non-cell systems [1–4, 7, 10–18].

The anti-inflammatory action of salicylate compounds is usually attributed to their effects on cyclooxygenase [19–21]. However, the salicylate metabolism studies cited above suggested that anti-inflammatory activity may also depend on metabolism of salicylates at sites of inflammation by activated inflammatory cells [7]. First, salicylates may preferentially undergo oxidative reactions thereby protecting host tissues [1]. Second, certain salicylate metabolites may themselves be anti-inflammatory. For example, 2,3-dihydroxybenzoate chelates iron [22], inhibits lipid peroxidation [23], and scavenges H_2O_2 [22, 23], and 2,5-dihydroxybenzoate is a potent inhibitor of prostaglandin synthesis [24, 25].

In the present study we report that activated neutrophils can hydroxylate salicylate to gentisic acid and 2,3-dihydroxybenzoic acid. The mechanism for this reaction was studied using antioxidant inhibitors and experiments with non-cell systems. Although the exact reaction remains uncertain, evidence is presented that gentisic acid is not produced by the myeloperoxidase (MPO) pathway.

MATERIALS AND METHODS

Reagents. Mannitol was obtained from Abbott Laboratories (North Chicago, IL). Deferoxamine was purchased from Ben Venue Laboratories (Bedford, OH). Phenol was from Mallinckrodt (Paris, KY). Sodium salicylate, gentisic acid, and all di-OH- and tri-OH-benzene compounds listed in Table 1 were purchased from the Aldrich Chemical Co. (Milwaukee, WI). The remainder of the compounds in Table 1 were purchased from the Sigma Chemical Co. (St Louis, MO). Catalase (C-40, 14,100 units/mg), superoxide dismutase (SOD, 2725 units/mg), azide, cyanide, 3-aminotriazole and phorbol myristate acetate (PMA) were purchased from Sigma. PMA was dissovled in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°. NaOCl was purchased from Aldrich and HOCl titered as previously described [26]. Purified human MPO was obtained from the Green Cross Corp. (Osaka, Japan).

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‖ Abbreviations: 5-ASA, 5-aminosalicylic acid; OH·, hydroxyl radical; O_2^- superoxide; H_2O_2, hydrogen peroxide; NaOCl, sodium hypochlorite; HOCl, hypochlorous acid; PMA, phorbol myristate acetate; MPO, myeloperoxidase; SOD, superoxide dismutase; KCN, potassium cyanide; GSH, glutathione; 3AT; 3-aminotriazole; DMSO, dimethyl sulfoxide; and DPBS, Dulbecco's phosphate-buffered saline.

Cell purification. Blood was obtained from normal volunteers who had been medication-free for at least 48 hr. Neutrophils were separated by dextran sedimentation and Ficoll–Hypaque density gradient centrifugation [27]. Hypotonic lysis was used to remove residual red cells. The purity of the granulocyte suspensions was >98%.

Production of hydroxylated salicylates. Neutrophils (20×10^6 cells/ml, final concentration) were placed in Dulbecco's phosphate-buffered saline (DPBS) containing 50 mg/dl glucose in Falcon 2059 tubes (Oxnard, CA). Sodium salicylate was added to yield a final concentration of 10 mM. In the inhibitor experiments, small volumes of the inhibitory compounds were added at this time. The tubes were capped with rubber stoppers and preincubated with stirring at 37° for 15 min, after which neutrophils were stimulated with 100 ng/ml PMA. The final concentration of DMSO was 0.7 mM. The same volume of DPBS was added to unstimulated controls, making a total volume of 1.5 ml in each tube. After 1 hr of incubation, tubes were chilled and then centrifuged (27,000 *g*) at 4° for 10 min. Supernatant fractions were maintained at −80° until analysis by HPLC (see below). To reduce the chance of chemical or biological contamination, new sterile disposable tubes and pipets were used for all solutions, incubations, and collections of supernatant fractions.

HPLC. The measurement of hydroxylated salicylates was performed by a modification of a previously described HPLC procedure for benzoate compounds [8]. The chromatograph was a Hewlett–Packard model 1084 B dual pump system operated in isocratic mode. The mobile phase was 5% aqueous acetic acid with 2 g/l of ammonium acetate (pH = 3.0), and the flow rate was 1.2 ml/min. The column was an IBM C-18, 4.5 mm × 25 cm, 5 μm that was preceded by a C-18, 10 × 4.6 mm, 5 μm, Nucleosil guard column (AllTech, Deerfield, IL). Absorbance was monitored at 240 and 278 nm using a variable wavelength detector.

Standard curves of gentisic acid were prepared daily by adding gentisic acid to the mobile phase (final concentrations of 0, 5, 10, 25, 50, 80 and 100 ng/50 μl) and injecting 50 μl into the HPLC. Plots of peak height (mAU at 240 nm) versus gentisic acid concentration were linear ($r > 0.999$). A typical response factor was 0.096 mAU/ng gentisic acid on column.

Cell-free supernatant fractions (50 μl) were analyzed directly (i.e. unextracted) by HPLC. Gentisic acid was quantified by peak height at 240 nm using the standard curve. Standards (100 ng each) of other potential metabolites of salicylic acid were injected into the HPLC to determine retention times and response factors at 240 or 278 nm (see Table 1).

HOCl *determination.* Hypochlorous acid (HOCl) production was quantitated by the chlorination of taurine as previously described [26]. Neutrophils (20×10^6 cells/ml, final concentration) were stimulated with PMA or zymosan at 37° as described above except that 15 mM taurine was added to the medium to trap HOCl. Reactions were terminated at 1 hr by icing and by the addition of 25 μg/ml of catalase. Following centrifugation (27,770 *g*, 20 min), the cell-free supernatant fractions were assayed for taurine chloramine by the I^- method [26, 28]. MPO activity was determined by a spectrophotometric assay that measures the oxidation of orthodianisidine substrate at 460 nm [28].

Statistics. Data are reported as mean ± SD. The effects of inhibitors were analyzed using the paired *t*-test (CLINFO). A P value < 0.05 was considered to be statistically significant.

RESULTS

Production of hydroxylated salicylates by activated neutrophils. PMA-stimulated neutrophils produced an obvious metabolite as detected in the 8-min peak at 240 nm (Fig. 1). Retention time analysis of gentisic acid standard and other possible metabolites in Table 1 indicated that the 8-min peak was gentisic acid. First, none of the other compounds in Table 1 had retention times in the 7.2 to 9.3 min range. Second, when the neutrophil supernatant fractions that contained the 8-min peak were spiked with standard gentisic acid, the standard co-eluted exactly with the 8-min peak. The 8-min gentisic acid peak was not observed under several control incubation conditions. These included cells + salicylate (no PMA), cells + PMA (no salicylate), PMA + salicylate (no cells), and salicylate alone (no cells, no PMA).

When 10 mM salicylate was present in the incubation, the production of gentisic acid by PMA-stimulated cells from fourteen normal volunteers averaged 55 ± 11 ng/10^6 cells (Table 2). This represented approximately 0.08% conversion of the starting salicylate to the gentisic acid metabolite. When salicylate was reduced to 1 mM in the same system, 13 and 10 ng of gentisic acid were formed in two separate experiments. No gentisic acid was observed when 0.1 mM salicylate was employed in the system.

PMA-stimulated supernatant fractions also contained a much smaller 2,3-dihydroxybenzoic acid peak eluting at approximately 12 min (Fig. 1). This was not produced in appreciable quantities in the control incubations. The 12-min retention time of the peak in the biologic sample was very close to the retention time of three dihydroxybenzoic acid compounds listed in Table 1 (the 2,3-, 2,4-, and 2,6-isomers). However, when the biologic sample was spiked with these compounds, the 2,3-dihydroxy compound co-eluted exactly with the 12-min peak whereas the other compounds produced separate peaks. The production of 2,3-dihydroxybenzoic acid by PMA-stimulated cells from six normal volunteers was 6.2 ± 2.7 ng/10^6 cells.

HPLC analysis of the PMA-stimulated supernatant fractions did not demonstrate other salicylate metabolites. For example, the retention times of standards of trihydroxybenzoic acids, resorcinol, catechol, phenol, and others (Table 1) did not correspond to the 8- and 12-min peaks in Fig. 1.

Neutrophil supernatant fractions were also monitored at 278 nm. At this wavelength, which is close to a λ minimum for gentisic acid, essentially no peak could be seen at 8 min. However, a single peak was consistently observed at 6 min. This compound remains unidentified.

Effects of antioxidants on gentisic acid formation. Because gentisic acid was easily quantified, it was

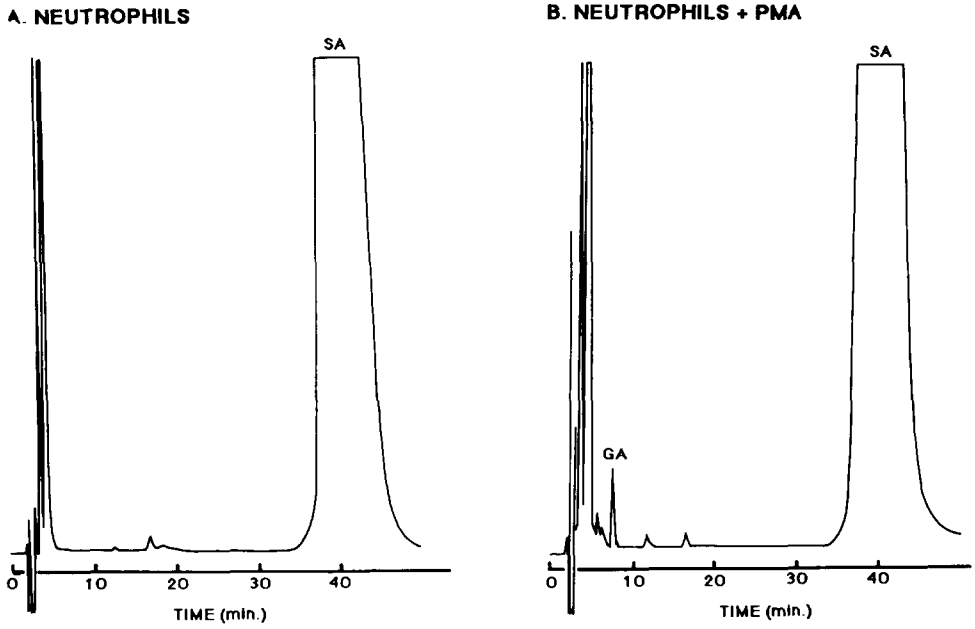

Fig. 1. HPLC analysis of hydroxylated salicylates. Neutrophils (30×10^6 total cells) were suspended in 1.5 ml of DPBS containing 10 mM salicylate (SA). Following preincubation at 37° for 15 min, cells were stimulated with PMA (100 ng/ml). After a 1-hr incubation the cell-free supernatant fractions were analyzed by HPLC at 240 nm (see Materials and Methods). The gentisic acid (GA) peak eluted at approximately 8.0 min. The smaller 12-min peak was 2,3-dihydroxybenzoic acid. A small 17-min peak was a contaminant in the salicylate.

Table 1. HPLC retention times of benzoic acid metabolites

Compound	Common name	Retention time* (min)	Number of determinations
Benzoic acid	—	63.3	1
Mono-OH-benzoic acid			
ortho-	Salicylic acid	39.26 ± 1.52	36
meta-	—	18.72 ± 0.34	3
para-	—	12.95 ± 0.20	4
Di-OH-benzoic acid			
2,3-		12.34 ± 0.26	12
2,4-	β-Resorcylic acid	12.49 ± 0.29	8
2,5-	Gentisic acid	8.04 ± 0.14	166
2,6-	γ-Resorcylic acid	11.79 ± 0.26	8
3,4-	Protocatechuic acid	7.14 ± 0.26	3
3,5-	α-Resorcylic acid	6.09 ± 0.19	5
Tri-OH-Benzoic acid			
2,3,4-	—	5.93 ± 0.14	10
2,4,6-	—	4.38	1
3,4,5-	Gallic acid	3.87†	1
Mono-OH-benzene	Phenol	21.70 ± 0.04†	2
Di-OH-benzene			
1,2-	Catechol	9.47 ± 0.18	3
1,3-	Resorcinol	6.64 ± 0.13†	6
1,4-	*p*-Benzoquinone	10.00 ± 0.14†	2
Tri-OH-benzene			
1,2,3-	Pyrogallol	4.29†	1
1,3,5-	Phloroglucinol	3.43†	1

Each compound (10 mg) was dissolved in 50 ml of acetonitrile and diluted to 2 ng/μl in mobile phase. Fifty microliter samples were injected into the HPLC (see Materials and Methods).

* Retention time was determined at 240 nm unless otherwise specified.

† Analyzed at 278 nm.

Table 2. Inhibition of gentisic acid formation by antioxidants

Condition	Gentisic acid (ng/10^6 cells)	N	% Inhibition*
Complete system (all experiments)	55 ± 11	14	—
Complete system without PMA (all experiments)	ND†	14	—
Complete system	51 ± 9	3	—
+ SOD (15 μg/ml)	4 ± 2‡	3	92
+ heat-inactivated SOD (15 μg/ml)	35 ± 8	3	32
Complete system	47 ± 15	4	—
+ catalase (100 μg/ml)	63 ± 17	4	−34
Complete system	52 ± 14	2	—
+ mannitol (40 mM)	49 ± 15	2	6
Complete system	57 ± 20	3	—
+ azide (0.1 mM)	4 ± 1‡	3	93
Complete system	52 ± 8	4	—
+ KCN (1 mM)	24 ± 11	3	54
+ KCN (5 mM)	5 ± 1‡	3	91
Complete system	52 ± 8	4	—
+ 3AT (0.5 mM)	42 ± 12	3	18
+ 3AT (10 mM)	3 ± 1‡	3	95
Complete system	67 ± 9	3	—
+ GSH (1 mM)	3 ± 2‡	3	96
Complete system	52 ± 2	3	—
+ deferoxamine (0.1 mM)	65 ± 4	3	−25
+ deferoxamine (1 mM)	87 ± 3	3	−67

The complete system consisted of 20×10^6 neutrophils/ml with 100 ng/ml phorbol myristate acetate (PMA) and 10 mM salicylate (see Fig. 1). Inhibitors were added to cells and salicylate prior to activation by PMA. Abbreviations: SOD, superoxide dismutase; KCN, potassium cyanide; 3AT, 3-aminotriazole; and GSH, glutathione.

* Calculated for each experiment as:

$$\frac{\text{gentisic acid (cells + PMA)} - \text{gentisic acid (cells + PMA + inhibitor)}}{\text{gentisic acid (cells + PMA)}} \times 100\%$$

† Non-detectable.

‡ Significant inhibition compared to control incubations, $P < 0.05$.

used to study the effects of several antioxidants on the hydroxylation reaction (Table 2). SOD (15 μg/ml) blocked 92% of gentisic acid formation and this inhibitory activity was largely destroyed by boiling the SOD for 15 min. This suggested a role for superoxide (O_2^-) or one of its products in the hydroxylation reaction. Catalase (100 μg/ml) did not inhibit gentisic acid formation. The hydroxyl (OH·) scavenger mannitol (40 mM) did not inhibit gentisic acid formation. This was true even when 1 mM salicylate was used so that a 40:1 ratio existed. Glutathione (1 mM) completely blocked gentisic acid formation.

All of the classic heme enzyme inhibitors, including azide, cyanide, and 3-aminotriazole, significantly inhibited gentisic acid formation (Table 2). These concentrations do not block the respiratory burst activity of neutrophils [9, 26]. These experiments indicated an apparent requirement for iron or a heme enzyme. However, the iron chelator deferoxamine did not inhibit and actually caused an increase in gentisic acid formation. Deferoxamine was greatly in excess of the concentration of free iron in our experiments. This was confirmed by measurements which showed no detectable iron in the cell-free supernatant fractions by the Ferene method [29], which has a lower limit of detectability of 2 μM. Thus, the hydroxylation of salicylate appeared not to be related to trace iron contamination in the incubation medium.

The inhibitory profile for 2,3-dihydroxybenzoic acid exactly paralleled that for gentisic acid. SOD, glutathione, azide, cyanide, and 3-aminotriazole blocked essentially all 2,3-dihydroxybenzoic acid formation (data not shown).

Evidence that the MPO pathway does not produce gentisic acid. The MPO pathway can metabolize salicylate compounds [2], and the inhibition of gentisic acid formation by azide, cyanide, 3-aminotriazole and glutathione in our study suggested a possible role for the MPO pathway. Several experiments were performed which did not support a role for MPO. First, we found that 10 mM salicylate incubated with reagent HOCl did not produce gentisic acid. Based on our prior work [28], PMA-stimulated neutrophils (20×10^6 cells/ml) can produce about 0.1 to 1.0 mM HOCl. Thus, we incubated 0.1 and 1.0 mM HOCl with 10 mM salicylate. Neither concentration of HOCl produced gentisic acid, although the 1.0 mM HOCl clearly metabolized salicylate and produced two later eluting peaks. Figure 2 illustrates these same findings using 0.1 mM salicylate with the two concentrations of HOCl. Thus, reagent HOCl does not produce gentisic acid.

We also studied the effect of the MPO-H_2O_2-Cl enzyme system on salicylate metabolism. Although Figure 2 had shown that reagent HOCl did not produce gentisic acid, we wanted to exclude reactions with other oxidants that might be produced as MPO generates HOCl. First, we incubated 0.3 mM H_2O_2

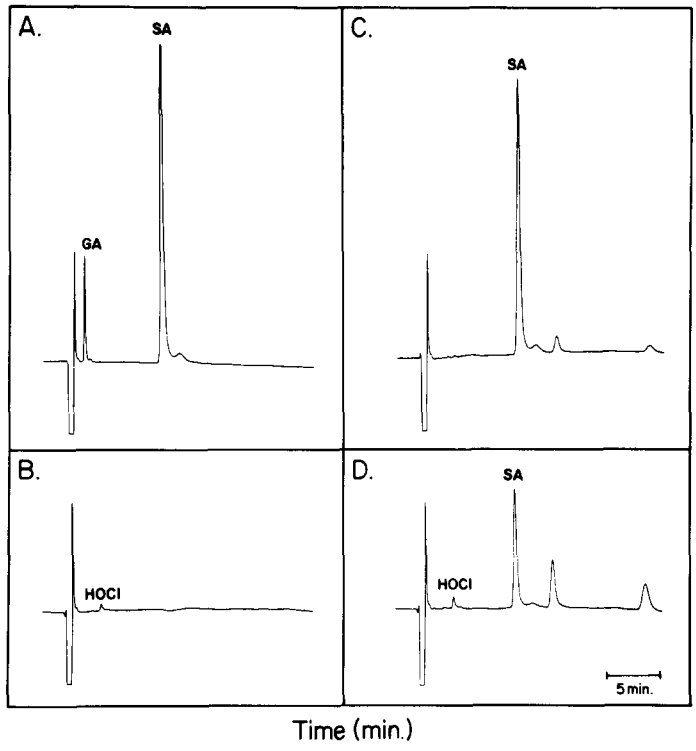

Fig. 2. HPLC analysis of reactions between salicylate and reagent HOCl. (A) Standards of gentisic acid (GA, 2 μg/ml) and salicylate (SA, 0.1 mM) dissolved in DPBS medium; (B) HOCl-derived peak produced by 1.0 mM HOCl in DPBS medium; (C) SA + 0.1 mM HOCl in DPBS medium; (D) SA + 1.0 mM HOCl. Note that no GA was produced in the reactions analyzed in (C) and (D), but that two new peaks eluting after SA were seen. The HPLC system differed from that mentioned in the legend of Fig. 1 and Materials and Methods in the use of aqueous ammonium acetate–acetonitrile–methanol (80%–10%–10%) and monitoring at 250 nm.

and 2 units/ml MPO in DPBS medium containing 15 mM taurine for 1 hr. We demonstrated formation of 0.27 mM HOCl, a concentration of HOCl equivalent to that produced by neutrophils in our system. We then repeated the incubation and allowed H_2O_2 and MPO to react simultaneously with 10 mM salicylate. Taurine was excluded. When analyzed by HPLC, no gentisic acid or 2,3-dihydroxybenzoic acid was formed (data not shown). Similar to Fig. 2, however, enough HOCl was formed to oxidize salicylate and produce later eluting peaks. Thus, the model MPO enzyme system did not produce gentisic acid.

Two other experiments were performed. First, in place of PMA, opsonized zymosan (5 mg/ml) was used to stimulate cells. Opsonized zymosan is a more effective secretagogue for MPO [3] and produces higher amounts of HOCl than does PMA [28]. However, we found that zymosan-stimulated cells produced only about 30% of the gentisic acid produced by PMA. Second, we reacted cell-free superantant fractions with salicylate. Neutrophils (20×10^6 cells/ml) were stimulated with PMA for 1 hr in DPBS medium without salicylate. Following centrifugation, the cell-free supernatant fractions were incubated for 1 hr with 10 mM salicylate. No gentisic acid was produced. This experiment did not support a role for chloramines, or "stable oxidants", in the generation of gentisic acid.

DISCUSSION

It has been known for many years that salicylate metabolism in humans produces hydroxylated salicylate compounds in serum and urine [30, 31]. Grootveld and Halliwell [32] recently reported 2,5-dihydroxybenzoate (gentisic acid) and 2,3-dihydroxybenzoate in the serum and synovial fluid of normal volunteers and rheumatoid arthritis patients taking aspirin. Although salicylate metabolism presumably occurs by liver microsomal pathways, it is possible that hydroxylation of salicylate also occurs at sites of inflammation by the action of inflammatory cells. The present report demonstrates that activated neutrophils can hydroxylate salicylate *in vitro*. Gentisic acid was the principal metabolite and was easily quantitated in the unextracted cell-free supernatant fractions. Other metabolites, including 2,3-dihydroxybenzoic acid, were observed. These studies add to the growing body of knowledge about drug metabolism by inflammatory cells [1–10, 33].

Oxidation reactions involving the salicylate family of compounds have been studied in various cell-free systems. Benzoate, the parent compound of the salicylate family, and salicylate react with OH· in radiolysis experiments [13–17]. Both drugs also react with OH· generated by xanthine-xanthine oxidase [3, 4, 7, 12]. Reaction of salicylate and 5-ASA with OH· generated by a Fenton system produces

hydroxylated salicylates which can be detected by HPLC [1, 11, 12, 32]. Benzoic acid can be decarboxylated by HOCl and MPO plus H_2O_2 [10]. Recent studies also demonstrate the ability of 5-ASA to react with hypochlorite and MPO plus H_2O_2 [2].

It is difficult to extrapolate findings in the non-cell system to the intact cell. Despite the use of various inhibitors and other experiments, we are uncertain about the exact mechanism for the production of hydroxylated salicylates by neutrophils. The hydroxylation reaction clearly occurs in association with respiratory burst activity triggered by PMA or zymosan. OH· released by activated neutrophils would seem to be a logical candidate for the hydroxylation reaction. Although OH· production by neutrophils has been questioned recently [3, 34, 35], this remains controversial, and a recent spin trapping study [36] supports OH· production through Haber–Weiss or Fenton type reactions requiring trace iron either inside or outside the cell. However, the failure of catalase, mannitol, and deferoxamine to inhibit salicylate hydroxylation in our study casts doubt on the importance of OH· released by these pathways. Another possible hydroxylation pathway is the MPO–H_2O_2-chloride enzyme system, since HOCl produced by a model MPO enzyme system can react with salicylate compounds [2]. Again, however, the antioxidant experiments in the present study are ambiguous and do not support an obvious role for the MPO enzyme system. Azide, 3-aminotriazole, cyanide, and glutathione blocked salicylate hydroxylation, whereas catalase, a univerally effective H_2O_2 scavenger, failed to inhibit. If MPO were important, SOD would have been expected to increase gentisic acid formation. In addition, we did not detect gentisic acid formation with relevant concentrations of HOCl, MPO plus H_2O_2, and cell-free supernatant fractions. Although all of these experiments are against a role for MPO, they do not absolutely rule out its importance in the intact cell. Finally, it remains possible that O_2^- or one of its products caused salicylate hydroxylation, since SOD effectively inhibited the reaction. However, since we have shown previously that salicylate does not interfere with O_2^- detection by the cytochrome *c* method [7], it is hard to understand how O_2^- alone could result in salicylate hydroxylation. For all these reasons, the exact oxidative pathway for salicylate hydroxylation by neutrophils is uncertain. It remains possible that the hydroxylation reaction is mediated by another oxidant species produced from O_2^-, for example, singlet oxygen ($^1O_2$). It is also possible that an unidentified enzyme system is responsible.

Our study is similar in some respects to the study of 5-ASA reported by Dull *et al.* [1]. Both studies reported the formation of gentisic acid by activated neutrophils in a SOD-inhibitable reaction. However, several differences were apparent. Starting substrates (salicylate vs 5-ASA) and stimuli (PMA vs cytochalasin B and chemotactic peptide) were different. Catalase had no effect in our system but blocked in their system. Also, no 2,3-dihydroxybenzoic acid was detected in their reactions, and the two products (gentisic acid and salicylate) represented deaminated products of 5-ASA. In their experiments it remains possible that MPO produced salicylate in a catalase-inhibitable reaction. Afterwards, the newly-formed salicylate was converted to gentisic acid in the same SOD-inhibitable reaction reported in our paper.

Salicylate hydroxylation can presumably occur by microsomal pathways in multiple tissues. Our results indicate that activated neutrophils can also hydroxylate salicylate. It is not clear what proportion of total salicylate hydroxylation occurs in individual tissues. Our results show that unstimulated neutrophils do not metabolize salicylate, suggesting that the contribution from this route is usually negligible. However, activated neutrophils clearly acquire the capacity to metabolize salicylate. It is tempting to speculate that this may explain why more gentisic acid is excreted in the urine of febrile patients taking aspirin compared to normals [30]. Grootveld and Halliwell [32] also hint that the hydroxylated compounds were present in higher concentrations in body fluids from rheumatoid arthritis patients compared to normals, although they admit that far too few patients were studied for this to be conclusive.

In summary, our results demonstrate that activated neutrophils can hydroxylate salicylate *in vitro*. The importance of the reaction *in vivo* depends on whether these metabolites have biologic activity at sites of inflammation. Whether recovery of hydroxylated salicylates from body fluids will prove useful for the study of inflammatory conditions remains to be determined.

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