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Bacterial Metabolism of Human Polymorphonuclear Leukocyte-Derived Arachidonic Acid

TANIA C. SORRELL,* MICHAEL MULLER, AND KRYSTYNA SZTELMA

Centre for Infectious Diseases and Microbiology, University of Sydney, Westmead Hospital, Westmead, New South Wales 2145, Australia

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Evidence for transcellular bacterial metabolism of phagocyte-derived arachidonic acid was sought by exposing human blood polymorphonuclear leukocytes, prelabelled with [^{3}H]arachidonic acid, to opsonized, stationary-phase *Pseudomonas aeruginosa* (bacteria-to-phagocyte ratio of 50:1) for 90 min at 37°C. Control leukocytes were stimulated with the calcium ionophore A23187 (5 μM) for 5 min. Radiochromatograms of arachidonic acid metabolites, extracted from A23187-stimulated cultures and then separated by reverse-phase high-performance liquid chromatography, revealed leukotriene B_4, its ω-oxidation products, and 5-hydroxyeicosatetraenoic acid. In contrast, two major metabolite peaks, distinct from known polymorphonuclear leukocyte arachidonic acid products by high-performance liquid chromatography or by thin-layer chromatography, were identified in cultures of *P. aeruginosa* with [^{3}H]arachidonic acid-labelled polymorphonuclear leukocytes. Respective chromatographic characteristics of these novel products were identical to those of two major metabolite peaks produced by incubation of stationary-phase *P. aeruginosa* with [^{3}H]arachidonic acid. Production of the metabolites was dependent upon pseudomonal viability. UV spectral data were consistent with a conjugated diene structure. Metabolism of arachidonic acid by *P. aeruginosa* was not influenced by the presence of catalase, superoxide dismutase, nordihydroguaiaretic acid, ethanol, dimethyl sulfoxide, or ferrous ions but was inhibited by carbon monoxide, ketoconazole, and 1,2-epoxy-3,3,3-trichloropropane. Our data suggest that pseudomonal metabolism of polymorphonuclear leukocyte-derived arachidonic acid occurs during phagocytosis, probably by enzymatic epoxidation and hydroxylation via an oxygenase. By this means, potential proinflammatory effects of arachidonic acid or its metabolites may be modulated by *P. aeruginosa* at sites of infection in vivo.

Eicosanoids released upon stimulation of phagocyte arachidonic acid metabolism by microorganisms are believed to be important mediators of the early host response to infection. Modulation of this response may be achieved in vivo by the process of transcellular metabolism, whereby arachidonic acid or a metabolite released from one cell type is taken up and metabolized by another. This process has been well described in hemopoietic cell populations (13). Several observations suggest that mammalian phagocytes and certain microorganisms are similarly capable of transcellular arachidonic acid metabolism. The export of arachidonic acid and its metabolites represents one of the secretory responses of the polymorphonuclear leukocyte (PMN) and/or mononuclear phagocyte to ingestion of *Toxoplasma gondii* (12), *Leishmania donovani* (22), and pyogenic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* or their exotoxins (2, 23–25, 27). Arachidonic acid taken up by viable *T. gondii* can be used as a substrate for production of monohydroxyeicosatetraenoic acids by mouse peritoneal macrophages during phagocytosis (12). *Pseudomonas* species and *E. coli* utilize several fatty acids as growth substrates, via β-oxidation to acetyl coenzyme A units (6, 18). Although not studied previously in *P. aeruginosa*, ω-oxidation of 6- to 10-carbon alkenes (21) and 8- to 18-carbon saturated fatty acids (11) and epoxidation of short-chain alkenes (14) have been described in the environmental pseudomonad *Pseudomonas oleovorans*.

The production of leukotrienes (leukotrienes B_4 and C_4 [LTB_4 and LTC_4, respectively]) by PMNs exposed to *P. aeruginosa* in vitro has been attributed to the release of phospholipase C (PLC) (heat-labile hemolysin) (2). PLC is an exoenzyme expressed by certain strains of *P. aeruginosa* during culture in minimal media lacking P_i (2, 26). In the present study, we used two pathogenic isolates of *P. aeruginosa* which had been cultured under conditions likely to suppress PLC production to seek evidence for bacterial metabolism of arachidonic acid released by human PMNs during phagocytosis.

MATERIALS AND METHODS

Neutrophil preparation. PMNs were separated from 30 ml of heparinized blood by centrifugation over 20 ml of Mono-Poly Resolving Medium (ICN/Flow Labs, Sydney, Australia). The PMN band was harvested and washed with calcium and magnesium-free Hanks' balanced salt solution. Contaminating erythrocytes were removed by brief hypotonic lysis (30 s in distilled water). PMNs of greater than 90% purity were resuspended in Hanks' balanced salt solution at a concentration of 5×10^6 cells per ml. Cells were labelled with 0.5 μCi of [5,6,8,9,11,12,13,15-^{3}H]arachidonic acid per ml (specific activity, 202.4 Ci/mmol; Amersham, Sydney, Australia) by incubation for 60 min at 37°C in a shaking water bath. Extracellular arachidonate (15 to 20% added radioactivity) was removed by washing in Hanks' balanced salt solution. Under these conditions, more than 99% of cell-associated radioactivity was present in the neutral lipid and phospholipid fractions of extracts prepared as described below and separated by normal-phase thin-layer chromatography (17). Less than 1% was present as arachidonic acid

* Corresponding author.

(data not shown). More than 95% of PMNs remained viable by trypan blue exclusion.

P. aeruginosa. Two clinical isolates of *P. aeruginosa* obtained from human blood were identified by standard criteria (7). Prior to use, bacteria were subcultured into nutrient broth (Oxoid, Basingstoke, United Kingdom). Stationary-phase *P. aeruginosa* cells were opsonized by incubation with heat-inactivated human serum (20%, vol/vol) in Dulbecco's modified Eagle's medium (DMEM; ICN/Flow Labs). In some experiments, 2.5×10^8 CFU of washed, stationary-phase *P. aeruginosa* was incubated with 0.5 μCi of [^{3}H]arachidonic acid in DMEM for 5 min at 37°C with shaking. Lipids were extracted before chromatography as described below. Since heat-labile hemolysin (PLC), an exoenzyme produced by *P. aeruginosa* under certain growth conditions (26), stimulated LTB_4 production by human PMNs (2, 15), we tested our isolates for heat-labile hemolytic activity (2) when cultured under our standard assay conditions. Briefly, 200 μl of bacterial supernatants from stationary-phase (20-h) pseudomonal cultures were incubated with 800 μl of a washed suspension of sheep erythrocytes (2%, vol/vol) in phosphate-buffered saline at 37°C for 60 min. Samples were then placed on ice. Intact erythrocytes were sedimented by centrifugation (2 min at 9,700 × *g*). The optical density of the supernatant was determined at 530 nm and expressed as a percentage of that in controls containing erythrocytes which had been lysed in distilled water. Hemolytic activity was also assayed in supernatants of stationary-phase pseudomonal cultures which had been boiled for 15 min (2). No heat-labile hemolytic activity was detected.

Stimulation of neutrophil arachidonate metabolism. [^{3}H]arachidonate-labelled PMNs (5×10^6) were incubated with 1 ml of serum-free DMEM containing opsonized *P. aeruginosa* (2.5×10^8 CFU) for up to 90 min or with 1 ml of DMEM containing 5 μM calcium ionophore A23187 for 5 min. Previous experiments had determined that phagocytosis was maximal at this bacterium/PMN ratio of 50:1. Reactions were terminated by the addition of 1 volume of ethanol.

Lipid extraction, separation, and identification. Lipids were extracted from cultures by the method of Bligh and Dyer (3), dried under nitrogen, and separated isocratically by reverse-phase, high-performance liquid chromatography (HPLC) by using an Ultrasphere C18 5-μm column (250 by 4.5 mm; Beckman Instruments, Sydney, Australia), a mobile phase of methanol-water-acetic acid (75:24.95:0.05, vol/vol/vol) adjusted to pH 5.4, and a flow rate of 1 ml/min (24). Radioactivity in 1-ml fractions was determined in a Packard liquid scintillation counter. The percentage of arachidonic acid metabolites in each of the radiochromatographic peaks was expressed relative to the total recoverable radioactivity (29). UV absorption spectra were determined on fractions containing chromatographic peaks of interest. The identities of LTB_4 and of 5-hydroxyeicosatetraenoic acid (5-HETE) in cultures of PMNs stimulated with the ionophore A23187 were confirmed by coelution with authentic standards on reverse-phase HPLC (mobile phase as described above), normal-phase thin-layer chromatography as described below (17), and by UV spectroscopy (4, 19).

The spectral data on each compound were consistent with published spectra. LTB_4 showed characteristic triene absorption maxima of 260, 270, and 281 nm (4); 5-HETE absorbed strongly at 235 nm (19). LTB_4 was also confirmed by radioimmunoassay (Amersham radioimmunoassay kit; data not shown).

In some experiments, HPLC fractions containing radioactive material of interest were reextracted, taken up in chloroform, and separated by thin-layer chromatography on Silica Gel G by using a mobile phase containing chloroform-methanol-acetic acid-water (90:8:1:0.8, by volume) (17). Slices (5 mm wide) were scraped from the plate, and radioactivity was determined as described above. Authentic standards of LTB_4, 5-HETE, and arachidonic acid were run in parallel with the products of interest.

Chemicals. A23187 (Boehringer Mannheim, Sydney, Australia), arachidonic acid (Sigma Chemical Co., St. Louis, Mo.), and nordihydroguaiaretic acid (NDGA; Sigma) were dissolved in ethanol. 1,2-Epoxy-3,3,3-trichloropropane (TCPO; Sigma) and ketoconazole (Janssen Pharmaceuticals) were dissolved in dimethyl sulfoxide. The concentration of ethanol or dimethyl sulfoxide in the final incubation mixtures was not greater than 0.1%. Catalase (Calbiochem, San Diego, Calif.), superoxide dismutase (Sigma), and $Fe_2SO_4 \cdot 7H_2O$ were diluted in sterile distilled water.

RESULTS

Metabolism of arachidonic acid in the presence of *P. aeruginosa*. Exogenous [^{3}H]arachidonic acid was metabolized by each clinical isolate of *P. aeruginosa* to two major products (P1 and P2) with respective HPLC elution characteristics similar to those of the two major products (P3 and P4) formed during phagocytosis of live, opsonized *P. aeruginosa* by [^{3}H]arachidonic acid-labelled PMNs (Fig. 1). Metabolites P1 and P3 (Fig. 1A and C) each coeluted with authentic standard LTB_4 and with LTB_4 produced by PMNs in response to A23187 (Fig. 1B). However, radioactive chromatographic peaks which coeluted with 5-HETE or with ω-oxidation products of LTB_4 were evident only in the cultures of PMNs stimulated with A23187 (Fig. 1B). These data suggested that PMN 5-lipoxygenase activity had not been stimulated by the pseudomonal isolates and that P3 and P4 were novel products of bacterial metabolism of neutrophil-derived arachidonic acid. The absence of LTB_4 from products P1 and P3 was confirmed by reextracting lipids from the HPLC fractions of interest and separating them by thin-layer chromatography. Figure 2 indicates that the products P1 and P3 (hereafter designated P1,3) coelute with each other but not with LTB_4 and that neither one contains LTB_4. The possibility that LTB_4 was formed early during the bacterium-PMN interaction was excluded by processing cultures at different times, 7.5 to 90 min after stimulation of PMNs with *P. aeruginosa*. No LTB_4 was detected (data not shown).

To further investigate the possibility of bacterial metabolism of arachidonic acid, the experiments with exogenous arachidonic acid and [^{3}H]arachidonic acid-labelled PMNs were repeated with opsonized *P. aeruginosa* which had been killed by exposure to heat. As shown in Fig. 3, none of the products P1,3 or P2,4 was formed in cultures containing killed *P. aeruginosa*.

We determined that each of the novel products absorbed UV light strongly at 235 nm and not at 269 nm. The UV spectrum of P1 revealed an absorption maximum of 236 nm, consistent with a conjugated diene structure. Insufficient material was available for analysis by gas chromatography-mass spectroscopy or by magnetic resonance spectroscopy.

Time course of arachidonic acid metabolism. The time course of [^{3}H]arachidonic acid metabolism in the presence of *P. aeruginosa* was studied in the absence and in the presence of PMNs. After exposure of *P. aeruginosa* to a pulse of [^{3}H]arachidonic acid in vitro, formation of the two novel products, P1 and P2, was complete within 45 s (Fig. 4).

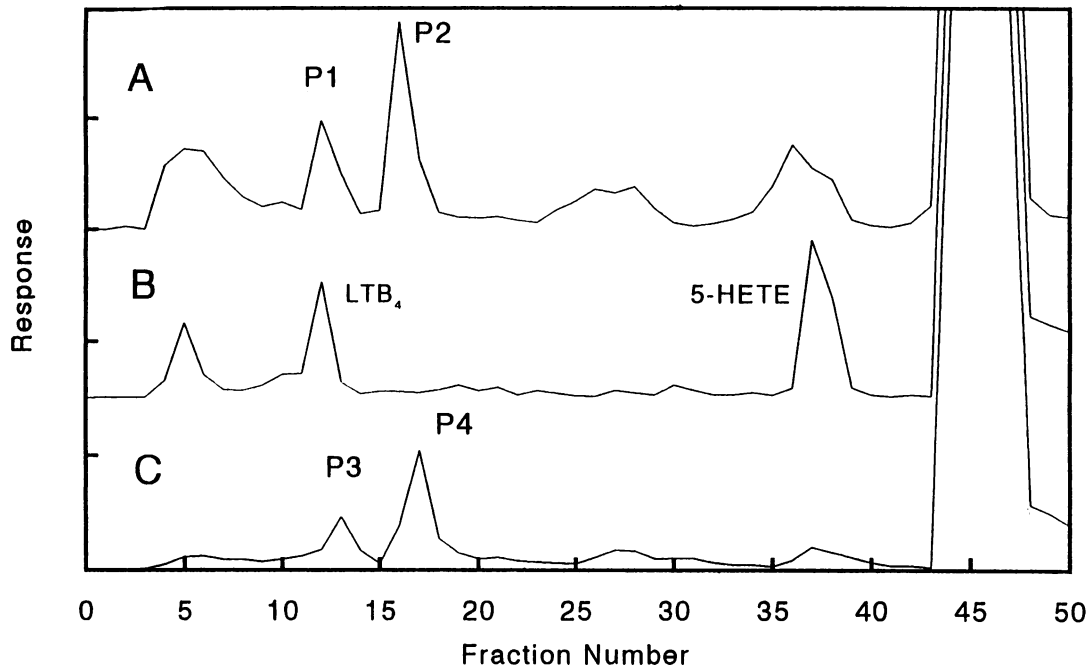

FIG. 1. Representative HPLC radiochromatograms of lipid extracts from *P. aeruginosa* incubated with [^{3}H]arachidonic acid (A), [^{3}H]arachidonic acid-labelled PMNs incubated with A23187 (B), and [^{3}H]arachidonic acid-labelled PMNs incubated with unlabelled, opsonized *P. aeruginosa* (C). In this system, ω-oxidation products of LTB_4 elute in fractions 4 to 6, LTB_4 elutes in fractions 11 to 13, 5-HETE elutes in fractions 36 to 38, and arachidonic acid elutes in the methanol wash (fractions 43 to 48).

When measured at 5 min, 52.9% ± 1.3% (n = 16, mean ± standard error of the mean) of the readily extractable radioactivity was present as polar products of arachidonic acid, the majority being identified in products P1 and P2 (Fig. 1A). These polar products were not metabolized during a further 6 h of incubation. Under the experimental conditions employed, saturation of the enzyme system(s) was not achieved. Thus, the quantity of products was directly pro-

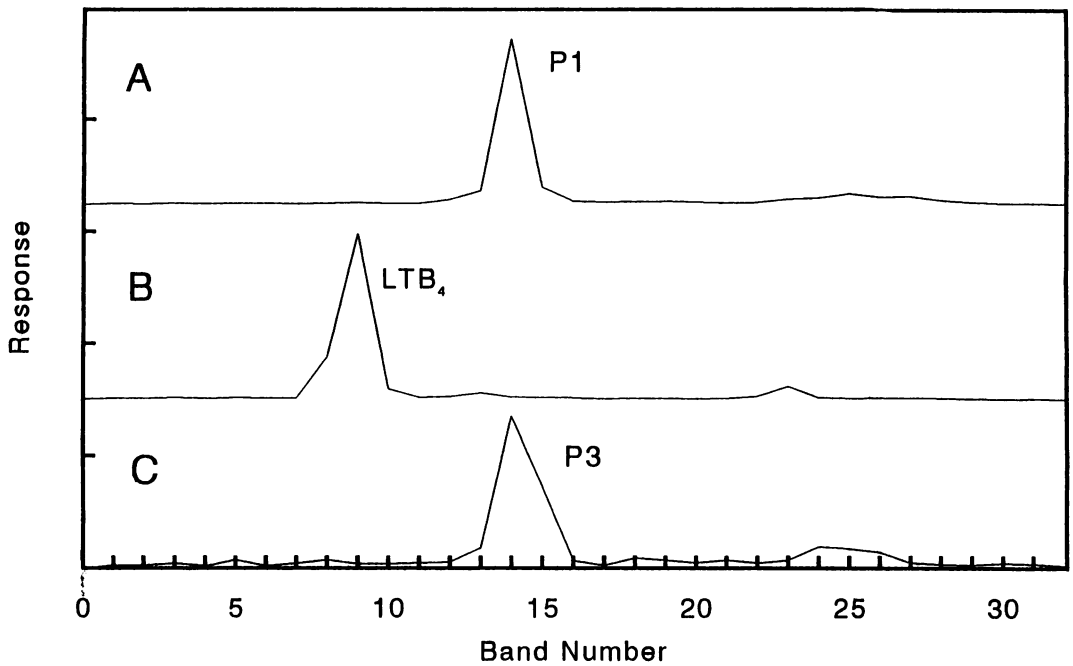

FIG. 2. Representative thin-layer chromatography radiochromatograms of product P1 produced by incubation of *P. aeruginosa* with [^{3}H]arachidonic acid (A), authentic LTB_4 (B), and product P3, produced by incubation of *P. aeruginosa* with [^{3}H]arachidonic acid-labelled PMNs (C).

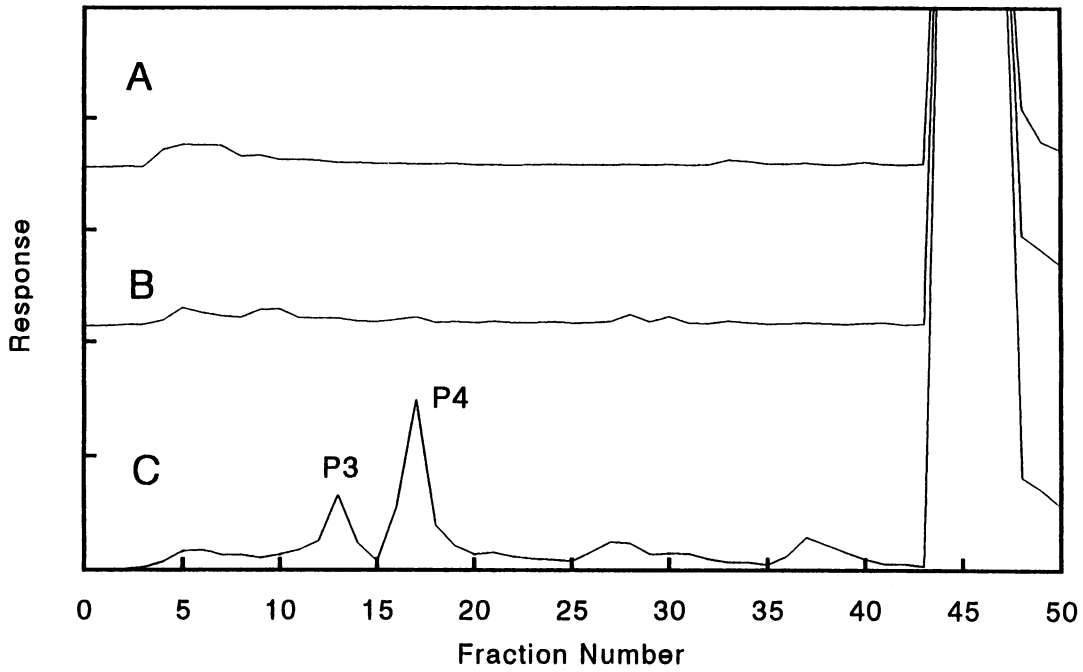

FIG. 3. Representative HPLC radiochromatograms of lipid extracts from cultures of opsonized, heat-killed *P. aeruginosa* incubated with [^{3}H]arachidonic acid (A) or with [^{3}H]arachidonic acid-labelled PMNs (B). Controls (C) contained viable *P. aeruginosa* incubated with [^{3}H]arachidonic acid-labelled PMNs.

portional to arachidonic acid concentrations of 2.4 to 7.2 nM, indicating that, at our standard concentration of 2.4 nM, the reaction was substrate limited. This was confirmed by pulsing the incubation mixture again with 2.4 nM arachidonic acid after completion of the initial reaction. The reaction rate and incremental increase in products remained constant.

In PMN-*P. aeruginosa*-containing cultures (Fig. 5), there

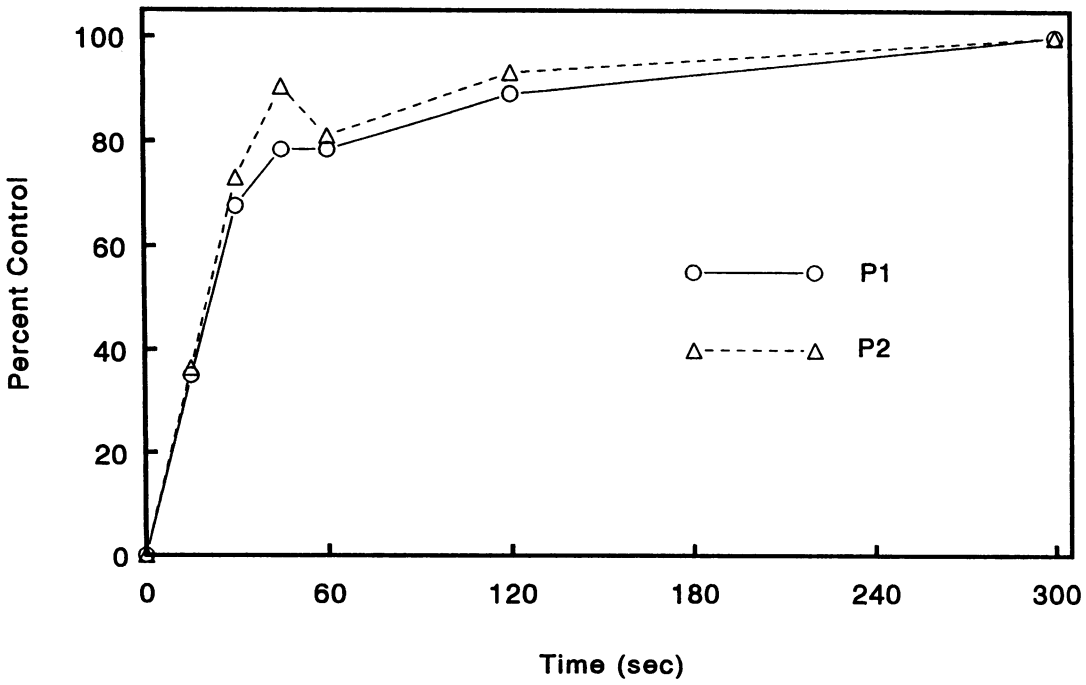

FIG. 4. Time course of arachidonic acid metabolism by *P. aeruginosa* showing formation of products P1 and P2 over the first 5 min of incubation. Radioactive label in P1 or P2 at each time point has been expressed as a percentage of the total radioactivity present in P1 and P2, respectively, after 5 min of incubation. Data from a representative experiment performed in duplicate are presented.

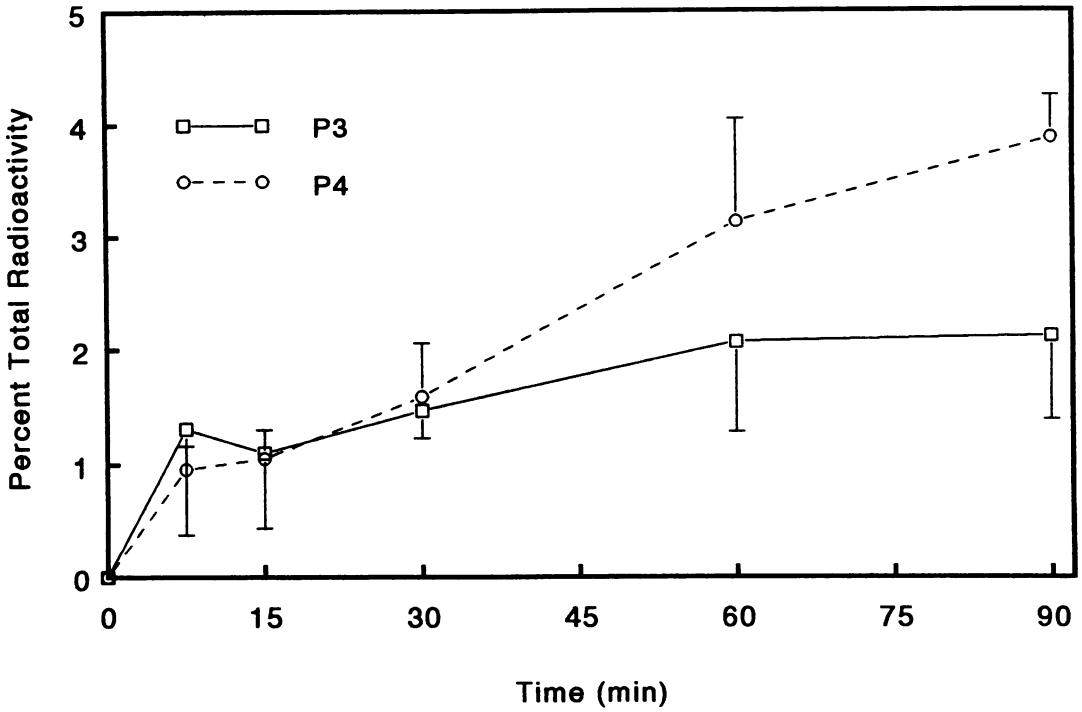

FIG. 5. Time course of metabolism of arachidonic acid to products P3 and P4 in [^{3}H]arachidonic acid-labelled PMN cultures incubated with viable *P. aeruginosa*. The percentage of radioactive label in each compound has been expressed as a percentage of the total recoverable radioactivity. Data points show means ± standard deviations for three experiments performed in duplicate.

was an early rapid rise in the amounts of products P3 and P4, consistent with the initial rapid release of arachidonic acid from PMN membrane phospholipid in response to a particulate stimulus and with bacterial metabolism of the free arachidonate. A subsequent slow accumulation of stable products was noted. After 60 min, 5.2% of the total radioactivity was present as polar products. This compares with the release of approximately 6% of the total radioactivity as LTB_4, its ω-oxidation products, and 5-HETE from PMNs stimulated for 5 min with A23187 (data not shown), a figure similar to that reported by others (29).

Metabolism of arachidonic acid by *P. aeruginosa*. Metabolism of [^{3}H]arachidonic acid was maximal in late-logarithmic- and early-stationary-phase bacteria. The effects of selected metabolic inhibitors, including carbon monoxide, ketoconazole, TCPO, and NDGA, are shown in Table 1. Nitrogen gas (N_2) was used as an anaerobic control for the CO studies (see Table 1). Catalase (10 μg/ml, 300 U), superoxide dismutase (10 μg/ml, 29 U), and the free radical scavengers dimethyl sulfoxide and ethanol (0.13 and 0.22 mM, respectively) were without effect. None of the inhibitors affected bacterial growth kinetics. To exclude iron-dependent peroxidation of arachidonic acid via a Fenton or iron-catalyzed Haber-Weiss reaction (1), *P. aeruginosa* was incubated with [^{3}H]arachidonic acid and different concentrations of Fe^{2+} (0 to 100 μM). There was no change in the amounts of products produced (data not shown).

DISCUSSION

Our experimental data are consistent with transcellular metabolism of human PMN-derived arachidonic acid by *P. aeruginosa*. Thus, the elution characteristics of the two major metabolites formed from arachidonic acid by *P. aeruginosa* (P1 and P2) were identical to *P. aeruginosa*-PMN-derived products (P3 and P4) on reverse-phase HPLC and normal-phase thin-layer chromatography, suggesting identity of P1 with P3 and P2 with P4. In addition, the initial time course of production of P3 was similar to that of P1, and that of P4 was similar to that of P2. Products P1,3 and P2,4 were identified only in cultures containing *P. aeruginosa*, and only chromatographically distinct PMN lipoxygenase products (LTB_4 and 5-HETE) were produced when PMNs were stimulated with A23187. The generation of the novel products was dependent absolutely on pseudomonal viability. We reported previously that novel arachidonic acid metabolites with HPLC elution characteristics similar to those found in this study were present in the supernatants of rabbit alveolar macrophage cultures after incubation with *P.*

TABLE 1. Effect of metabolic inhibitors on bacterial metabolism of arachidonic acid

Inhibitor (concn)[a]	% Inhibition of products[b]	
	P1	P2
CO-air[b]	83.6	84.9
N_2-air[b]	18.6	48.5
Ketoconazole (20 μM)	79.1 ± 2.9	84.5 ± 3.1
TCPO (1 mM)	80.5 ± 3.2	75.2 ± 5.7
NDGA (10 μM)	29.5 ± 9.3	1.0 ± 4.7

[a] For CO-air and N_2-air, media were bubbled through with CO or N_2 for 15 min prior to and for 5 min after addition of [^{3}H]arachidonic acid to bacteria. The CO/O_2 ratio was calculated to be 11:1 on the basis of the known solubility of CO in water. Other inhibitors were preincubated with bacteria for 20 min prior to adding [^{3}H]arachidonic acid.

[b] Mean ± standard error of mean. Two (CO-air and N_2-air) or three experiments were performed in duplicate.

aeruginosa and that pharmacological inhibition of macrophage cyclooxygenase and/or lipoxygenase activity had no effect on their production (24). Furthermore, in contrast to the findings with *P. aeruginosa*, another pyogenic bacterium, *S. aureus*, neither metabolized arachidonic acid nor elicited the formation of these products when incubated with rabbit alveolar macrophages (24). Microbial metabolism of phagocyte-derived arachidonic acid has not been reported by other workers, although Locksley et al. (12) noted that the obligate intracellular protozoan *T. gondii* incorporates, but does not metabolize, exogenous arachidonic acid and van Dyk et al. reported recently that the yeast *Dipodascopsis uninucleata* can metabolize arachidonic acid to 3-HETE (28).

Bergmann et al. (2) induced PLC production in four of nine strains of *P. aeruginosa* isolated from patients with severe burns by culture in phosphate-free medium. This enzyme activity was correlated with stimulation of LTB_4 and LTC_4 synthesis in human PMNs. Products consistent with those of bacterial origin were not observed. However, these workers monitored for leukotrienes by measuring UV absorbance at a wavelength of 280 nm; the products identified in our study absorbed well at 235 nm and were not evident when scanned at a wavelength suitable for detection of leukotrienes (i.e., 269 nm) (4).

The mechanism by which *P. aeruginosa* converts arachidonic acid of PMN origin to polar metabolites has not been fully elucidated. The inhibitor studies reported herein are consistent with oxidation via an ω-hydroxylase system. Thus, the reaction was inhibited substantially by carbon monoxide, which binds reversibly to ferrous cytochrome P-450 (30) and ketoconazole, an inhibitor of cytochrome P-450-dependent mixed-function oxidases in mammalian liver microsomes (30). The reaction was also inhibited by TCPO, an epoxide hydrolase inhibitor (20), suggesting that the oxidation of arachidonic acid proceeds through an epoxide intermediate(s). Two complex ω-oxidation systems have been described in pseudomonads: the cytochrome P-450-dependent system for metabolism of camphor in *Pseudomonas putida* (9) and the hexane-inducible mixed-function oxidase system of *P. oleovorans*. The enzyme in *P. oleovorans* hydroxylates the methyl group of alkanes and 8- to 18-carbon saturated fatty acids (11, 21), although 16- and 18-carbon fatty acids are oxidized more slowly than 8- to 14-carbon acids (11). This enzyme may also be responsible for epoxidation of terminal olefin bonds (14). The system in *P. aeruginosa* differs from that in *P. oleovorans* in being inhibited by carbon monoxide.

Catalase, superoxide dismutase, dimethyl sulfoxide, and ethanol were without effect on the two products, suggesting that metabolic conversion by peroxidation of arachidonic acid is unlikely. Iron-dependent peroxidation of arachidonic acid via an iron-catalyzed Haber-Weiss reaction was excluded by the Fe^{2+} dose-response studies, in which there was no effect of added iron on product formation. In addition, we found no evidence of peroxidation products in our radiochromatograms, assuming that on reverse-phase HPLC, coelution with peroxidation products known to be produced during incubation of *S. aureus* with arachidonate would be expected (10). The significance of the 30% inhibition of product P1 (with no effect on product P2) in the presence of NDGA is difficult to interpret. NDGA is a radical scavenger, lipoxygenase inhibitor (8), and inhibitor of cytochrome P-450 (5).

Previous studies, including our own, have shown that *P. aeruginosa* can utilize arachidonic acid for growth, presumably via activation of a thiokinase(s) and β-oxidation (6). In the present experiments, glucose was present as an alternative carbon source and approximately 50% of the readily extractable ^3H was converted into stable polar products. Although the identity of these products has not been established, their chromatographic profile, their strong UV absorption at 235 nm, and the inhibitor studies are consistent with conjugated dienes, possibly dihydroxylated metabolites of arachidonic acid. Large-scale production of pure products is necessary for definitive identification, and development of the methodology is currently under way.

P. aeruginosa is an important pathogen in hospitalized patients and in immunocompromised hosts, with a high mortality. Our finding that *P. aeruginosa* modulates PMN utilization of arachidonic acid via transcellular metabolism is consistent with a bacterial antiinflammatory effect and complements our recently published observation that the pseudomonal phenazine pigments, pyocyanin and 1-hydroxyphenazine, inhibit production of neutrophil 5-lipoxygenase products (16).

These antiinflammatory properties of *P. aeruginosa* may be potentially important virulence factors, which should be taken into account in models of pathogenesis of pseudomonal disease and which may be amenable to therapeutic intervention.

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