Identification of a Novel Arachidonate 12-Lipoxygenase in Bovine Tracheal Epithelial Cells Distinct from Leukocyte and Platelet Forms of the Enzyme*

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We examined the characteristics of an arachidonate 12-lipoxygenase in bovine tracheal epithelial cells in relation to the enzyme expressed in leukocytes and platelets. Homogenous preparations of intact or disrupted tracheal epithelial cells metabolized arachidonic acid predominantly to (12S)-hydroxyeicosatetraenoic acid, and subcellular fractionation by differential centrifugation demonstrated that the 12-lipoxygenase activity was localized predominantly to the 100,000 x g supernatant (cytosol fraction). Analysis of cytosolic enzymatic activity for pH dependence (maximum activity at pH 7.4-8.0), divalent cation effects (no dependence on cations), and kinetic characteristics (lag phase elimination by addition of hydroperoxide) exhibited similarity to leukocyte and platelet 12-lipoxygenases. Immunoprecipitation experiments demonstrated that the epithelial 12-lipoxygenase reacted with a monoclonal antibody (lox-2) directed against leukocyte 12-lipoxygenase but not with an antibody (HPLO-3) against the platelet enzyme. Immunoonaffinity chromatography of the epithelial 100,000 x g supernatant fraction using lox-2 linked to Affi-Prep 10 yielded a single predominant protein band (Mr = 72,000) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis identical in apparent mass to the bovine leukocyte lipoxygenase. Western blotting using a polyclonal antibody to leukocyte 12-lipoxygenase showed peroxidase staining of the same 72-kDa protein band. Activity assays of the purified enzymes demonstrated that substrate specificity for the epithelial 12-lipoxygenase was similar to that of the leukocyte enzyme, but the epithelial enzyme more efficiently converted 18-carbon fatty acids to the corresponding monohydroxylated conjugated dienes. We conclude that bovine tracheal epithelial cells express a 12-lipoxygenase that has immunological reactivity similar to leukocyte and distinct from platelet 12-lipoxygenase and possesses substrate specificity distinct from both enzymes. We further suggest that lipoxygenase heterogeneity may provide a basis for different functional roles for the enzyme in different cell types.

The arachidonate 12-lipoxygenase was the first of the lipoxygenases to be discovered in mammalian tissues. The enzyme was described initially in platelets (1, 2) and then later in porcine and bovine polymorphonuclear leukocytes (3-5) and murine eosinophils (6). It catalyzes the reaction of arachidonic acid with molecular oxygen to form (12S)-hydroperoxyeicosatetraenoic acid (12-HPETE),1 which, under most conditions, is reduced to the corresponding hydroxy fatty acid (12-hydroxyeicosatetraenoic acid, 12-HETE) and released from the cell. The precise function of 12-lipoxygenase activity remains uncertain, but a number of the enzymatic products have been reported to have potent effects as specific mediators in a variety of tissues (reviewed in Ref. 7).

The proposed heterogeneity of 12-lipoxygenase function may be explained by the hypothesis that the enzyme serves different functional roles in different cell types. That possibility is supported by evidence of biochemical heterogeneity among 12-lipoxygenases isolated from leukocytes versus platelets. Initial studies of bovine and porcine lipoxgynases demonstrate that the leukocyte form of the enzyme exhibits a much broader range of substrate acceptability in comparison with the platelet enzyme (2, 5, 8). More recently, the 12-lipoxygenases from bovine leukocytes and platelets have also been distinguished from one another by immunoprecipitation with distinct monoclonal antibodies (9). Furthermore, the immunoonaffinity-purified enzyme from platelets shows little activity for octadecapolyenoic acid substrates relative to the leukocyte enzyme.

To define the capabilities of lipid mediator generation in the pulmonary airway epithelium, we have investigated the capacity of isolated bovine tracheal epithelial cells to metabolize arachidonic acid. We chose to study bovine cells in view of the well characterized bovine platelet and leukocyte lipoxygenases and thereby aimed to avoid possible species differences (10). We discovered that the predominant arachidonic acid metabolite from bovine tracheal epithelial cells was (12S)-HETE (11), implicating the action of an arachidonate 12-lipoxygenase. The present experiments were designed to

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1 The abbreviations used are: HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; PG, prostaglandin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGF2α, [ethylenebis(oxyethylenenitrilo)j etraacetic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
characterize the enzyme definitively using immunoprecipitation and immunofluorimetry chromatography with anti-12-lipoxygenase monoclonal antibodies and to determine whether the epithelial enzyme differed in its immunological or kinetic characteristics from the leukocyte and platelet forms of the enzyme. The results represent the first reported purification and characterization of an epithelial cell lipoxygenase.

EXPERIMENTAL PROCEDURES

Sources of Materials—[1-14C]Arachidonic acid (54.9 mCi/mmol), [18O]arachidonic acid (95.1 Ci/mmol), and [1-14C]linoleic acid (50.5 mCi/mmol) were from Du Pont-New England Nuclear; unlabeled arachidonic, linoleic, α-linolenic, α-linolenic, 11,14,17-ecisatrienoic, 8,11,14-ecisatrienoic, and 4,7,10,13,16,19-docosahexaenoic acid were from NuChek Prep Inc. (Elysian, MN); 5,8,11,14,17-ecisapentaenoic acid, Type I collagenase, Type XIV protease (ProTase 2E), Triton X-100, and Tween 20 were from Sigma; protein A-bearing Staphylococcus aureus and anti-mouse IgG were from Boehringer Mannheim; trimethylphosphite was from Aldrich; organic solvents were from Riedrich and Jackson Laboratories Inc. (Muskegon, MI); and phosphate-buffered saline and Hanks’ balanced salt solution were from Grand Island Biological Co. (Lincoln, NE). Other reagents were of the highest purity available.

Preparation of Tissues—Rat tracheal mucosa was obtained from tracheal segments obtained from New Zealand White male rats (200-250 g) that were killed by decapitation, and respiratory epithelial enzyme differed in its immunological or kinetic characteristics from the leukocyte and platelet forms of the enzyme. The results represent the first reported purification and characterization of an epithelial cell lipoxygenase.

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lial cells or leukocytes (5–15 ml) was applied. The column was washed with equilibration buffer for 10 min at 1 ml/min, and then enzyme was eluted with 50 mM diethanolamine (pH 10.4) containing 0.3% Triton X-305, 0.5 mM NaCl, 20% glycerol, 2 mM 2-mercaptoethanol, and 20 μM ferrous ammonium sulfate. Fractions of 1 ml each were collected in tubes containing 0.2 ml of 1.0 M Tris-HCl (pH 7.4), assayed for 12-lipoxygenase activity and protein concentration, and stored at -70 °C.

**Immunoblotting**—Aliquots of the cytosol fraction from sonicated epithelial cells and leukocytes and from immunofluorescence-purified 12-lipoxygenase from leukocytes and epithelial cells were subjected to 10% polyacrylamide gel electrophoresis (PAGE, 1-mm thickness) in the presence of 0.1% sodium dodecyl sulfate (SDS) (14). Protein bands on the gel were detected by silver staining. The gels also underwent electrophoretic transfer to nitrocellulose membranes (15), which were then stained with a monoclonal rabbit antibody against the porcine leukocyte 12-lipoxygenase (16). Binding of primary antibody was detected using a biotinylated anti-rabbit IgG secondary antibody and a preformed avidin-biotinylated horseradish peroxidase complex (Vectorstain ABC kit, Vector Laboratories). The membrane was washed and subjected to the peroxidase reaction by exposure to a mixture of 0.6 mM 3,3′-diaminobenzidine HCl, 3 mM hydrogen peroxide, and 50 mM Tris-HCl (pH 7.6).

**Spectrophotometric Assay of Purified Enzyme**—An aliquot (40–100 μl) of the eluate from immunofluorescence chromatography containing leukocyte or epithelial 12-lipoxygenase activity was added to 120–160 μl or 800–900 μl of 200 mM Tris-HCl (pH 7.4) (final reaction volume of 200 μl or 1 ml) containing 25 μM fatty acid substrate, 0.02% Tween 20, and 1 μM 13-HPODE at 37 °C. The reaction time course of the purified 12-lipoxygenase was monitored continuously by detection of conjugated dienes at 240 nm in a Beckman DU-64 spectrophotometer, and initial velocity of the reaction was calculated for each substrate using a molar absorption coefficient of 22,000. Assays were monitored at 240 nm (rather than 235 nm) because of a decrease in background absorbance and consequent improvement in signal/noise.

**RESULTS**

**Identification of Whole Cell and Cytosol Products**—Bovine tracheal epithelial cells and the 100,000 × g supernatant fraction from disrupted cells invariably converted arachidonic acid to a single predominant metabolite comigrating with 12-HETE during reverse-phase HPLC (Fig. 1). Analysis of the product by gas chromatography and electron-impact mass spectrometry (11) and by chiral-phase HPLC (Fig. 2) demonstrated that the compound was (1S)-HETE. Generation of 12-HETE was detected only when metabolites were processed and analyzed at -10 °C by TLC (Fig. 3). In parallel experiments (not shown), bovine polymorphonuclear leukocytes and the 100,000 × g supernatant fraction from disrupted leukocytes generated a nearly identical profile of metabolites during analysis by reverse-phase HPLC. Mean levels of 12-HETE were 601 and 158 pmol/106 epithelial cells and leukocytes, respectively (n = 9). Both epithelial and leukocyte cytosol fractions incubated with arachidonic acid also resulted in the generation of a low level of (1S)-HETE (Figs. 1 and 2).

**Characterization of Enzyme Preparation**—Initial experiments demonstrated that the epithelial 12-lipoxygenase activity was relatively confined to the 100,000 × g supernatant (cytosol) fraction of disrupted cells. Mean specific activity for the cytosolic fraction incubated with a concentration of arachidonic acid (80 μM) resulting in maximal 12-HETE formation was 13.8 ± 4.9 nmol/30 min/mg of protein (n = 8). Enzymatic activity in the 12,000 × g pellet (mitochondrial fraction) and the 100,000 × g pellet (microsomal fraction) was insignificant (<0.5 nmol/30 min/mg of protein). This pattern of subcellular localization is similar to that reported previously for the bovine leukocyte and platelet 12-lipoxygenases that were isolated and assayed under similar conditions (5, 9).

Other characteristics of the epithelial lipoxygenase evident from assay of the cytosol fraction of disrupted cells included: (i) the lack of dependence on divalent cations; (ii) the presence of a lag phase that was eliminated by the addition of hydrogen peroxide; and (iii) maximum enzymatic activity at pH 7.4–8.0. Enzymatic activity as assessed by 12-HETE generation was not increased by the addition of 5 mM calcium, magne-

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**FIG. 1.** Reverse-phase HPLC analysis of radiolabeled arachidonic acid products released from bovine tracheal epithelial cells (A) and from 100,000 × g supernatant (cytosol) fractions from disrupted epithelial cells (B). Whole cells (6 ml of 2 × 106 cells/ml of Heps-buttered Hanks’ balanced salt solution, pH 7.4) or the cytosol fraction (2 ml of 0.3 mg of protein/ml of 100 mM Tris-HCl, pH 7.4) were incubated with [3H]arachidonic acid diluted with unlabeled arachidonic acid (final concentrations of 80 μM arachidonic acid and 2.2 × 106 dpm/ml) for 30 min at 37 °C. Products were extracted into acidic chloroform or diethyl ether and then resolved using the acetonitrile/water/acetic acid system described under “Experimental Procedures.” Major peaks of radioactivity eluted with 12-HETE and arachidonic acid (AA) and smaller peaks with PGE2 and 15-HETE.

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**FIG. 2.** Chiral-phase HPLC analysis of UV-absorbing (A) and radiolabeled (B) arachidonate products released from the cytosol fraction of disrupted bovine tracheal epithelial cells. The cytosol fraction (diluted in 2 ml of 100 mM Tris-HCl to a final concentration of 0.3 μg of protein/ml) was incubated with [14C]arachidonic acid for 30 min at 37 °C. Products were extracted into acidified diethyl ether, converted to the corresponding methyl esters, and analyzed by chiral-phase HPLC using the hexane/2-propanol solvent system described under “Experimental Procedures.”
clonal antibodies that selectively precipitate bovine leukocyte lipoxygenase was tested with two anti-12-lipoxygenase monoclonal antibodies based against the porcine leukocyte 12-lipoxygenase lox-2 and one against the human platelet 12-lipoxygenase HPOLO-3. Antibodies were linked to protein A-bearing S. aureus for lox-2 and to protein A plus anti-mouse IgG for HPOLO-3. Varying amounts of antibody were incubated with the 100,000 x g supernatant (cytosol) from sonicated epithelial cells (200 µl of 3 mg protein/ml of Tris HCl (pH 7.4)). The immune complex was precipitated, and the supernatant was assayed for enzymatic activity using TLC as described in Fig. 3. Assay of resuspended lox-2 immune complexes revealed 12-lipoxygenase activity that varied inversely with the corresponding supernatant activity and increased quantitatively with amount of lox-2 used (data not shown). Control experiments confirmed that HPOLO-3 quantitatively immunoprecipitated bovine platelet 12-lipoxygenase. 

Peaks of radioactivity correspond to trioxilin Aa and B, (peak 1), hepoxilin A, and B3 (peak 2), 12-HETE, 12-HPETE, and arachidonic acid (AA).

The immune complex was precipitated, and the supernatant contained lox-2 antibody linked to Affi-Prep 10, and the chromatography resulted in marked purification of the epithelial 12-lipoxygenase. The enzyme was eluted by increased pH, detergent, and salt concentrations, and evidence of purification was obtained by detection of enzymatic activity in only selected fractions (Fig. 5). Yields of purified epithelial enzyme were 4-6% of initial activity applied to the column; the corresponding yield for leukocyte enzyme was 15%. Total protein recovered from the column in the fractions containing 12-lipoxygenase activity was 1% of the initial total protein applied for both leukocyte and epithelial cell preparations. 

The specific activities of the purified epithelial and leukocyte enzymes based on initial reaction velocity with arachidonic acid were 0.3 and 0.7 µmol/min/mg of protein at 37°C, respectively, and were comparable to values reported previously (9).

Analysis of the epithelial lipoxygenase-containing fractions by SDS-PAGE and silver staining revealed a single predominant protein band (M, = 72,000) (Fig. 6). The molecular mass was identical to leukocyte 12-lipoxygenase purified under identical conditions. Furthermore, Western blot analysis of the immunoaffinity-purified epithelial and leukocyte enzymes using a polyclonal anti-12-lipoxygenase also stained a single protein band of identical mass (Fig. 7).

Substrate Utilization—Initial reaction velocities of immunoaffinity-purified leukocyte and epithelial enzyme were tested in parallel to compare substrate specificity of the two enzymes. Reaction rates of the purified enzyme were determined by continuous spectrophotometric assay in the presence of Tween 20 and a hydroperoxy acid to normalize kinetic properties of the 12-lipoxygenase (17). In general, the two enzymes were active with a similar range of fatty acid substrates. Both enzymes exhibited significant activity for a variety of eicosapolyenoic acid substrates; however, the epithelial enzyme exhibited even greater activity than the leukocyte lipoxygenase for 18-carbon fatty acid substrates (Table I).

To confirm that characteristics of the purified enzymes were representative of the 12-lipoxygenase activities contained in epithelial cells and leukocytes, the cytosolic fraction from each cell type was assayed for activity with a radiolabeled
12-Lipoxygenase in Tracheal Epithelial Cells

FIG. 6. SDS-PAGE of immunoaffinity-purified 12-lipoxygenase from bovine tracheal epithelial cells and polymorphonuclear leukocytes. Enzyme purification was carried out as described in Fig. 5. The immunoaffinity-purified enzymes were subjected to SDS-PAGE in 10% polyacrylamide, and a predominant protein band at 72 kDa was detected by silver staining.

FIG. 7. Western blot of bovine leukocyte and epithelial cell 12-lipoxygenase. SDS-PAGE was carried out as described in Fig. 6, and samples then underwent electrophoretic transfer to nitrocellulose and polyclonal anti-12-lipoxygenase antibody staining with 3,3'-diaminobenzidine as a peroxidase substrate. Lanes 1 and 3 contain purified epithelial cell lipoxygenase (1 × and 0.5 ×, respectively), and lanes 2 and 4 contain purified leukocyte lipoxygenase.

18-carbon fatty acid substrate (linoleic acid) relative to arachidonic acid. Over a wide range of substrate concentrations, the cytosolic fraction prepared from epithelial cells exhibited markedly higher activity for linoleic acid than did the corre-

TABLE I

<table>
<thead>
<tr>
<th>Fatty acid substrate</th>
<th>Initial velocity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Epithelial</th>
<th>Leukocyte</th>
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<tbody>
<tr>
<td>8,11,14,17-Eicosatetraenoic (arachidonic)</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>9,12-Octadeadienoic (linoleic)</td>
<td>51 ± 11</td>
<td>10 ± 4</td>
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<tr>
<td>9,12-Octadecatrienoic (γ-linolenic)</td>
<td>72 ± 18</td>
<td>17 ± 7</td>
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<tr>
<td>9,12,15-Octadecatrienoic (α-linolenic)</td>
<td>46 ± 9</td>
<td>21 ± 5</td>
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<tr>
<td>11,14-Eicosadienoic</td>
<td>29 ± 5</td>
<td>24 ± 6</td>
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<tr>
<td>4,7,10,13,16,19-Docosahexaenoic</td>
<td>91 ± 14</td>
<td>88 ± 8</td>
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</tbody>
</table>

<sup>a</sup> Values represent percent of activity for arachidonic acid (mean ± S.E., n = 3–5 experiments).
<sup>b</sup> 100% activity was equivalent to 4.6 and 5.0 nmol/min for epithelial and leukocyte enzymes, respectively.

FIG. 8. Concentration-response curves for 12-lipoxygenase activity in cytosol fractions from bovine tracheal epithelial cells (A) and bovine leukocytes (B). Fractions (diluted in 100 mM Tris-HCl and 0.02% Tween 20 to final protein concentrations of 0.6–0.8 mg of protein/ml) were incubated with [13C]arachidonic acid (O) or linoleic acid (●) for 30 min at 37 °C, extracted with acidic diethyl ether, and analyzed by HPLC as described in Fig. 1. Products identified as 12-HETE (from arachidonic acid) and 13-HODE (from linoleic acid) were quantified using specific activity. Each value represents the mean of duplicate samples.

FIG. 9. Western blot of bovine leukocyte and epithelial cell 12-lipoxygenase. SDS-PAGE was carried out as described in Fig. 6, and samples then underwent electrophoretic transfer to nitrocellulose and polyclonal anti-12-lipoxygenase antibody staining with 3,3'-diaminobenzidine as a peroxidase substrate. Lanes 1 and 3 contain purified epithelial cell lipoxygenase (1 × and 0.5 ×, respectively), and lanes 2 and 4 contain purified leukocyte lipoxygenase.

18-carbon fatty acid substrate (linoleic acid) relative to arachidonic acid. Over a wide range of substrate concentrations, the cytosolic fraction prepared from epithelial cells exhibited markedly higher activity for linoleic acid than did the corre-

DISCUSSION

The present data demonstrate that bovine tracheal epithelial cells contain an archidonate 12-lipoxygenase that exhibits immunological reactivity and kinetic characteristics similar to the leukocyte and distinct from the platelet form of the enzyme. In particular, the epithelial enzyme was quantitatively immunoprecipitated and immunoaffinity purified using the same monoclonal antibody (lox-2) as for the bovine leukocyte 12-lipoxygenase. By contrast, epithelial lipoxygenase showed no reactivity with an antiplatelet 12-lipoxygenase (HPLO-3). Furthermore, characterization of substrate lipoxygenation of the purified enzymes demonstrated that epithelial and leukocyte 12-lipoxygenase exhibit relatively broad substrate specificity in comparison with the platelet form of the enzyme (2, 5, 8, 9). In fact, the epithelial enzyme showed...
further differences in rates of substrate lipoxygenation even from the leukocyte form of the enzyme. It is therefore possible that subtle differences in enzyme structure are present between even the leukocyte and epithelial 12-lipoxygenases, but definitive proof of further heterogeneity will require characterization of enzyme structures.

The presence of an active 12-lipoxygenase in epithelial cells was unexpected. Immunohistochemistry and immunohistochemistry of 12-lipoxygenase in porcine tissues demonstrate the enzyme in leukocytes of peripheral blood (13) and various organs (16) but not in resident parenchymal cells (16). Previous studies of isolated tracheal (and nontracheal) epithelial cells from other species have also failed to detect predominant 12-lipoxygenase activity (10, 12, 18). Of note is the presence of a highly active arachidonate 15-lipoxygenase in epithelial cells from human trachea, and this enzyme shares many of the biochemical features of the bovine tracheal epithelial 12-lipoxygenase (10–12). Both lipoxygenases convert arachidonic acid to 15- and 12-HETE (although in differing proportions), and this dual capability enables both to generate an identical series of dihydroxylated conjugated triene derivatives (3, 9, 10, 12, 17, 19). For other fatty acid substrates, even the predominant product of the two lipoxygenases is the same. For example, both enzymes convert linoleic acid to its 13-hydroperoxy analogue (8, 20). It is therefore of interest that linoleic acid is the most abundant substrate for oxygenation in tracheal epithelial cells (21) and might serve as a major fatty acid source of oxygenated mediators of cell function. The high concentrations of the 12- and 15-lipoxygenases in airway epithelial cells and granulocytes (13, 19, 22) continue to suggest a role for the lipoxygenases in the development of allergic and inflammatory hypersensitivity (7).

The present characterization of a distinct epithelial 12-lipoxygenase may have other implications for lipoxygenase function. Of particular interest are the findings of relatively broad substrate acceptability for the epithelial form of the 12-lipoxygenase and the relatively evanescent detection for some of its lipoxygenation products (e.g. hydroperoxy and hydroxyeicosanoic acid). These findings suggest that the enzyme may play a much broader role in fatty acid modification than generation of only arachidonate-derived mediators. Furthermore, even for arachidonic acid, oxygenation products other than the stable mono- and diHETEs might be expected to have biological effects. It is also possible that differences in enzyme-substrate interaction between lipoxygenases from different cell types may confer a degree of specificity which is critical to their role in cellular function. For example, the capacity of epithelial lipoxygenase to utilize a variety of fatty acid substrates might make the enzyme a candidate to serve as a safeguard against the array of fatty acids released by epithelial phospholipase activity (10, 21). This function may not be as vital in leukocytes or platelets if they have little release of 18- or 20-carbon polyenoic fatty acids or if the cells possess alternative mechanisms for elimination of excess or unsuitable fatty acid substrates.

Acknowledgment—We gratefully acknowledge the expert technical assistance of Barbara Fordman and Laurie Prude.

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