A Cryptic, Microsomal-type Arachidonate 12-Lipoxygenase Is Tonicly Inactivated by Oxidation-Reduction Conditions in Cultured Epithelial Cells*

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Cultured ovine tracheal epithelial cells converted arachidonic acid to prostaglandin E₂ (PGE₂), but microsome-containing subcellular preparations were converted arachidonic acid to PGE₂ and to 12-hydroxyicosatetraenoic acid (12-HETE) at a high rate (2–4 nmol/mg of protein/15 min). Identification of the membrane-bound 12-HETE-forming activity as a 12-lipoxygenase included 12S-stereospecificity of product formation and trapping of 12-hydroperoxyicosatetraenoic acid as a reaction product. The 12-lipoxygenase activity was extracted from cell membranes only with detergent (1% Triton X-100), and the activity (membrane-bound or detergent-solubilized) was completely inactivated by mixing with the cytosol-containing subcellular fraction. The inhibitory effect of the cytosolic fraction was reversed by treating the cytosol with GSH-depleting agents (2-cyclohexene-1-one or N-ethylmaleimide) or by mixing it with lipid hydroperoxide (13-hydroperoxyoctadecadienoic acid) at a concentration that had little direct effect on enzyme activity. Inhibition of 12-lipoxygenase activity could also be achieved by treatment of enzyme preparations with GSH at levels (0.1–10 mm) found in epithelial cell cytosol. In addition, treatment of cultured epithelial cells with a GSH-depleting agent (buthionine sulfoximine) and lipid hydroperoxide restored cellular 12-lipoxygenase activity. Little or no detectable 12-lipoxygenase activity was found in freshly isolated ovine tracheal epithelial cells, but the cytosolic 12-lipoxygenase found in freshly isolated bovine tracheal epithelial cells was relatively insensitive to regulation by GSH or lipid hydroperoxide. These observations indicate that a 12-lipoxygenase is expressed in a cryptic, microsomal-type form in primary-culture epithelial cells and that this form of the enzyme may be selectively regulated by changes in cellular oxidation-reduction conditions dependent on cytosolic levels of GSH versus lipid hydroperoxide.

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Experimental Procedures

Materials—[1-14C]Arachidonic acid (54.9 mCi/mmol), [1H]arachidonic acid (95.1 Ci/mmol), and [1-14C]linoleic acid (52.1 mCi/mmol) were from Du Pont-New England Nuclear; unlabeled arachidonic acid was from NuChek Prep Inc. (Elysian, MN); collagen (vitrogen) was from Celsic Laboratories (Palo Alto, CA); and other chemical reagents and drugs were from Sigma. LHC basal medium was obtained from Biofluids (Rockville, MD) and was supplemented with bovine pituitary extract, epidermal growth factor, epinephrine, hydrocortisone, insulin, L-glutamine, transferrin, triiodothyronine, calcium chloride (0.11 mM), trace elements, penicillin (100 units/ml), and streptomycin (100 µg/ml).
Electron microscopy demonstrated that microsomes were contained in HEp-2 epithelial cells, which can synthesize PG, prostaglandin. These conditions were designed to maximize recovery of lipoxygenase after 20 min and 100,000 g for 200 s in a disruption buffer of 50 mM EDTA, 1 mM EGTA, 5-80 μM [3H]arachidonic acid, and 1% Triton X-100 (total volume of 2 ml). Assays were carried out at 37°C for 2-45 min after addition of enzyme. Preliminary experiments indicated that maximal lipoxygenase activity was achieved by incubation with 10-20 μM arachidonic acid and that the activity increased linearly over the range of total protein concentrations from 0.1-0.5 mg/ml. In some experiments, the monoclonal-containing or cytosolic subcellular fractions were assayed after treatment with 13-HPODE or with N-ethylmaleimide or 2-cyclohexene-1-one (GSH-derivatizing agents) at concentrations that provide maximal activity (23-25). In comparative experiments, freshly isolated ovine or bovine tracheal epithelial cells were prepared, subjected to fractionation, and assayed for oxygenation activity using identical procedures.

HPLC Analysis of Oxidation Products—Cells and enzyme mixtures were extracted with 2-propanol/acetic acid/chloroform or diethyl ether/acidic acid using PGE₂ as an internal standard for product recovery as described previously (26, 27). Extracts were reconstituted in chromatographic solvent and analyzed by reverse phase HPLC as described previously (27). For reverse-phase HPLC, the chromatograph was fitted with a 4.6 × 100 mm analytical column packed with 3-μm octadecylsilane-coated particles and was run at a flow rate of 1.0 ml/min with two solvents (A and B) that were 95% acetonitrile/1.5% triethylamine and 10% triethylamine/1% acetic acid respectively. The outflow from the spectrophotometer was monitored using a diode array spectrophotometer at 270 nm for conjugated trienes (including PGE₂ and 235 nm for conjugated dienes (including 12-HETE). The outflow from the spectrophotometer was routed to a radioactivity detector for concurrent measurement of ³H or ¹⁴C. Compounds were quantified using standard molar absorption coefficients and measurements of specific activity (28).

TLC Analysis of Products—Enzyme incubations with [¹⁴C]arachidonic acid were stopped with the addition of 2.25 volumes of diethyl ether, methanol, 1 M citric acid (30:4:1), the mixture was centrifuged at 1500 × g, and the upper phase was spotted on 20 × 20-cm Silica Gel 60 plates which were developed in petroleum ether/diethyl ether/acetic acid (15:85:0.1, v/v) for 50 min at -10°C and then subjected to autoradiography for detection of products. TLC reference standards for 12-HETE and 12-HETE were generated by incubation of [¹³C]arachidonic acid with bovine epithelial 12-lipoxygenase as described previously (13).

Assay of GSH Level—Cellular GSH content was determined by methods developed by Tietze (28) and Griffith (23) and later modified by Liang et al. (29). Briefly, 100 μl of cell cytosal was mixed with 800 μl of 0.3 mM NAPDH in 125 mM sodium phosphate, pH 7.5, with 6.3 mM EDTA, 100 μl of 6 mM 5,5'-dithiobis(nitrobenzoic acid), and 20 μl of 50 units/ml GSH reductase. The mixture was incubated at 30°C, and the change in absorbance at 412 nm was used to calculate total GSH concentration by comparison to a standard curve. Oxidized GSH (GSSH) was determined using the same method after first derivatizing endogenous GSH with 4-vinlypyridine. Cytosolic GSH content was calculated as the difference between total and oxidized GSH levels.
Evidence for a Lipoxygenase Mechanism for 12-HETE Formation in Membranes from Cultured Epithelial Cells—The type of enzyme responsible for 12-HETE formation by epithelial cell membranes was established by determining the degree of enzymatic stereospecificity (detection of 12S- versus 12R-HETE) and the requirement for hydroperoxide generation (detection of 12-HPETE). Absolute stereochemistry of 12-HETE was assigned by coelution of epithelial cell-derived 12-HETE with authentic standards under assay conditions: analysis of the stereochemistry of the 12-HETE generated from epithelial cell membranes demonstrated that it consisted entirely of the 12S isomer (Fig. 2A). Formation of 12-HPETE was determined by comigration of epithelial cell-derived 12-HPETE with authentic standards under assay conditions (incubation for 15 min at 25 °C and analysis of products by TLC at 4 °C) which permit detection of the labile hydroperoxide intermediate (13); analysis of radiolabeled arachidonate metabolites generated from epithelial cell membranes demonstrated that arachidonate was converted to 12-HPETE (Fig. 2B). The initial 12-lipoxygenase-catalyzed abstraction of the 10S-hydrogen of arachidonic acid followed by typical insertion of molecular oxygen on the opposite face of the substrate molecule at carbon-12 (antarafacial addition) leads to the formation of 12S-HPETE (30). Thus, our findings indicate that 12-HETE derived from cultured ovine tracheal epithelial cells is formed by the activity of an arachidonate 12-lipoxygenase and not by other HETE-forming enzymes such as cytochrome P-450 monoxygenases that typically generate a mixture of R- and S-enantiomers and do not form an obligatory hydroperoxide intermediate (27).

Evidence for 12-Lipoxygenase Sensitivity to Cytosolic Inhibition That Is GSH-dependent—The results of subcellular fractionation studies (Table I) suggested the presence of a cytosolic inhibitor of the epithelial microsomal 12-lipoxygenase. Verification of a cytosolic inhibitory effect was obtained when recombining the microsome-containing subcellular fractions (12,000 or 100,000 × g pellets) with the cytosolic fraction that the activity is microsomal; because the 12,000 × g pellet (under the present conditions for cell disruption) contains microsomes and other cellular organelles, localization of 12-HETE-forming activity in other cellular membranes (e.g. mitochondrial) cannot be excluded.

Additional experiments using increasing concentrations of detergent (0.01–5% Triton X-100) to extract the membrane-bound enzyme indicated that the 12-HETE-forming activity was completely extracted from epithelial cell membranes only with 1% Triton X-100 and that progressive increases in activity were accompanied by corresponding increases in extracted total protein (not shown). These results suggest that the 12-HETE-forming enzyme is an integral membrane protein. In addition, the fact that the cell disruption and fractionation experiments and the enzyme assays were performed in the presence of divalent cation chelation suggests that the enzyme responsible for 12-HETE formation is bound to the microsomal membrane by a non-calcium-dependent mechanism.

In contrast to the results with cultured ovine tracheal epithelial cells, experiments with freshly isolated ovine tracheal epithelial cells showed no evidence of a membrane-bound 12-HETE-forming activity (and only low and often undetectable levels of cytosolic 12-HETE-forming activity). It is therefore likely that the cell culture conditions used for the present experiments were responsible for induction of the microsomal 12-HETE-synthetic activity in cultured ovine tracheal epithelial cells (see below).

Evidence for a Lipoxygenase Mechanism for 12-HETE Formation in Membranes from Cultured Epithelial Cells—The type of enzyme responsible for 12-HETE formation by epithelial cell membranes was established by determining the degree of enzymatic stereospecificity (detection of 12S- versus 12R-HETE) and the requirement for hydroperoxide generation (detection of 12-HPETE). Absolute stereochemistry of 12-HETE was assigned by coelution of epithelial cell-derived 12-HETE with authentic, stereochemically pure 12-HETE enantiomeric standards during chiral-phase HPLC (13, 27); analysis of the stereochemistry of the 12-HETE generated from epithelial cell membranes demonstrated that it consisted entirely of the 12S isomer (Fig. 2A). Formation of 12-HPETE was determined by comigration of epithelial cell-derived 12-HPETE with authentic standards under assay conditions (incubation for 15 min at 25 °C and analysis of products by TLC at 4 °C) which permit detection of the labile hydroperoxide intermediate (13); analysis of radiolabeled arachidonate metabolites generated from epithelial cell membranes demonstrated that arachidonate was converted to 12-HPETE (Fig. 2B). The initial 12-lipoxygenase-catalyzed abstraction of the 10S-hydrogen of arachidonic acid followed by typical insertion of molecular oxygen on the opposite face of the substrate molecule at carbon-12 (antarafacial addition) leads to the formation of 12S-HPETE (30). Thus, our findings indicate that 12-HETE derived from cultured ovine tracheal epithelial cells is formed by the activity of an arachidonate 12-lipoxygenase and not by other HETE-forming enzymes such as cytochrome P-450 monoxygenases that typically generate a mixture of R- and S-enantiomers and do not form an obligatory hydroperoxide intermediate (27).
resulted in decreased 12-lipoxygenase activity (Table II). Combining the microsome-containing fractions with the cytosolic fraction in the same proportions as those isolated from the cell suspension (1:1, v/v) resulted in significant inhibition of 12-lipoxygenase activity, and mixing the membrane-bound 12-lipoxygenase with varying amounts of the cytosolic fraction resulted in a concentration-dependent alteration of activity (Table II). The same inhibitory effect of cytosol on 12-lipoxygenase activity was observed when detergent-solubilized enzyme (instead of resuspended microsomal membranes) was tested (not shown).

Inhibition of 12-lipoxygenase activity was also achieved by treatment of membrane-bound 12-lipoxygenase with GSH (Table II) in a range of concentrations (0.1–16 mM) equivalent to those found in cultured ovine tracheal epithelial cells (see below) as well as a variety of other cell types and tissues (23–25, 29, 31, 32). Interestingly, the PGH synthase/PGE isomerase activities of cultured tracheal epithelial cells were increased (rather than decreased) by GSH and were markedly decreased (not insensitive) to lipid hydroperoxide treatment (Table II). Thus, the net effect of cytosol on the PGH synthase/PGE isomerase pathway was to increase activity, in contrast to the cytosolic inhibition of microsomal 12-lipoxygenase activity. To test whether the microsomal 12-lipoxygenase sensitivity to GSH was distinct, we performed comparative experiments with the cytosolic 12-lipoxygenase which is expressed in freshly isolated tracheal epithelial cells from several animal species (33). Because freshly isolated ovine tracheal epithelial cells were a poor source of cytosolic 12-lipoxygenase activity (as noted above), we utilized freshly isolated bovine tracheal epithelial cells to prepare cytosolic 12-lipoxygenase as described previously (13). The cytosolic 12-lipoxygenase exhibited only slight inhibition by the highest concentrations of GSH or cytosol prepared from cultured ovine tracheal epithelial cells (Table II). Taken together, these findings imply that the membrane-bound 12-lipoxygenase expressed in cultured ovine tracheal epithelial cells is selectively susceptible to inhibition by a GSH-dependent mechanism.

GSH and GSH peroxidase are capable of reducing hydrogen peroxide and organic hydroperoxides, but direct evidence for regulation of oxidation-reduction metabolism by GSH in vivo is often lacking (22). Accordingly, we first tested the effects of the lipid hydroperoxide 13-HPODE and glutathione-depleting agents on the cytosolic inhibition of epithelial microsomal 12-lipoxygenase activity. Treatment of the cytosolic fraction with 13-HPODE, at a concentration that abolished the lag phase of the 12-lipoxygenase reaction (13, 34) and had little direct effect on the final level of 12-lipoxygenase activity (Table II and Fig. 3A), was capable of markedly reversing the cytosolic inhibition of the activity (Table III and Fig. 3B). It was also possible to mimic the 13-HPODE-dependent reversal of cytosolic inhibitory activity by treating the cytosol with the glutathione-derivatizing agents 2-cyclohexene-1-one or N-ethylmaleimide (Table III). The effect of N-ethylmaleimide (which may be less specific than 2-cyclohexene-1-one) (24, 25) was due to derivatization of GSH, because the subsequent addition of 10 mM GSH to the assay buffer reversed the capacity of N-ethylmaleimide to restore 12-lipoxygenase activity (Table III). These findings suggested that the cytosolic inhibition of microsomal 12-lipoxygenase activity depended on the endogenous balance between formation of lipid hydroperoxides (which stimulate the 12-lipoxygenase activity) and GSH-dependent depletion of the hydroperoxides (which leads to inhibition of the 12-lipoxygenase). GSH depletion or 13-HPODE treatment of the cytosolic fraction from cultured

![Fig. 2. Chiral-phase HPLC analysis of [14C]12-HETE (A) and TLC analysis of [14C]12-HPTE (B) derived from epithelial cell 12-lipoxygenase. (A) Microsome-containing fractions (12,000 and 100,000 x g pellets) prepared from cultured tracheal epithelial cells were incubated with 20 μM [14C]arachidonic acid for 15 min at 37 °C; products were extracted into acidified diethyl ether, and analyzed by a hexane/2-propanol solvent system. Major peaks of radioactivity coeluted with 12S-HETE (125) with a smaller amount of 15S-HETE (155) and no detectable 12R-HETE (12R). In (B), the microsome-containing fractions from cultured epithelial cells were incubated in increasing amounts (60, 15, and 4 μg of protein in lanes 1, 2, and 3) in 100 mM Tris-HCl with 25 μM [14C]arachidonic acid for 15 min at 25 °C. Products were extracted into acetylated ether, and analyzed by TLC at −10 °C using a petroleum ether/diethyl ether/acetic acid solvent system. Radioactive bands correspond to 12-HPETE (12H), 12-HETE (12H), and arachidonic acid (AA).](image-url)
epithelial cells resulted in a concomitant increase in cytosolic 12-lipoxygenase activity (final ratio of cytosolic/microsomal 12-lipoxygenase specific activities of 1–2.1), indicating that the epithelial 12-lipoxygenase may be expressed in membrane-bound and soluble-cytosolic forms.

Direct evidence that modulation of cellular GSH and hydroperoxide levels influenced 12-lipoxygenase activity in vivo was finally provided when GSH depletion and lipid hydroperoxide supplementation of cultured epithelial cells markedly increased 12-HETE synthetic activity (Table IV). Treatment of cultured ovine tracheal epithelial cells with buthionine sulfoximine (an irreversible inhibitor of γ-glutamylcysteine synthase) caused significant decreases in cellular GSH levels, increases in 12-HETE-forming activity, and magnified increases in activity evoked by lipid hydroperoxide. The pretreatment levels of GSH in cultured ovine tracheal epithelial cells (7.8 nmol GSH/10^7 cells) were in the middle of the range determined for other cell types and tissues (23–25, 29, 31, 32).

The base-line value for GSH in cultured epithelial cells corresponds to an intracellular GSH concentration of 3.9 mM based on an intracellular water volume of 2 μl/10^7 cells (35) and is well within the range of GSH concentrations used in the present experiments to inhibit microsomal 12-lipoxygenase activity (see above). The relatively small effect of GSH depletion alone (without hydroperoxide supplementation) on epithelial 12-lipoxygenase activity (Table IV) may reflect the difficulty in depleting intracellular stores of GSH (and other reducing agents) to a level that results in significant hydroperoxide tone (36). In comparative experiments, GSH depletion (by treatment with 2-cyclohexene-1-one, N-ethylmaleimide, or buthionine sulfoximine) of freshly isolated ovine tracheal epithelial cells caused no significant increase in 12-HETE-forming activity, and therefore (as noted above) provided no evidence for a cryptic 12-lipoxygenase (cytosolic or microsomal) in freshly isolated ovine cells. These findings again imply that the microsomal-type 12-lipoxygenase is selectively sensitive to GSH-dependent inhibition and selectively induced in cultured ovine tracheal epithelial cells.

In summary, the present characterization of arachidonate oxygenases in cultured ovine tracheal epithelial cells indicates that 12-HETE formation is catalyzed by a 12-lipoxygenase. An unusual feature of this epithelial 12-lipoxygenase is that it may be membrane-bound. Thus, 12-lipoxygenase activity is more generally confined to the cytosolic fraction (e.g. in porcine or bovine granulocytes or in freshly isolated bovine tracheal epithelial cells) (1, 5, 13, 34). Even when the 12-lipoxygenase is detected in membranes (e.g. in platelets), the localization may be highly dependent on increases in intracellular calcium concentration (37). The present observation for distribution of 12-lipoxygenase to the microsome-containing subcellular fractions under calcium-free conditions suggests an additional mechanism for enzyme localization in cultured epithelial cells. Our finding that the 12-lipoxygenase expressed in cultured tracheal epithelial cells is completely extracted from the membrane only with 1% Triton X-100 detergent suggests that this form of the enzyme may contain a hydrophobic domain which allows for alternative membrane localization. Purification studies are underway to determine the difference between this form of the epithelial 12-lipoxygenase and the previous cytotoxic forms described in tracheal epithelial cells, leukocytes, and platelets (33).

In addition to its unusual cellular location, the microsomal type 12-lipoxygenase in cultured epithelial cells exhibits an unusual sensitivity to inactivation by the cytosolic fraction. A similar sensitivity to cytosolic inhibition was recently observed for a membrane-bound 12-lipoxygenase in A431 epidermoid carcinoma cells, but the mechanism for the inhibitory effect was not determined (38). The present findings indicate

**TABLE IV**

Stimulatory effect of GSH depletion and lipid hydroperoxide supplementation on epithelial cell 12-lipoxygenase activity

| Cell treatment | GSH | Specific activity |
|----------------|-----|------------------|
|                |     | 12-Lox PG syn/isom |
| None           | 3.89| <0.01 2.24       |
| BSO (0.2 mM)   | 0.54| 0.17 2.36        |
| BSO (1 mM)     | 0.46| 0.17 2.20        |
| 13-HPODE (10 μM) | ND | 1.49 1.18  |
| BSO (1 mM)/13-HPODE (10 μM) | ND | 2.18 1.08 |

* Values represent the mean for two experiments.
that the cytosolic inhibition in cultured tracheal epithelial cells depends at least in part on the endogenous balance between formation of lipid hydroperoxides which stimulate 12-lipoxygenase versus GSH-scavenging of the hydroperoxides which serves to inhibit the enzymatic activity. The present findings do not exclude the possibility that other factors (besides alterations of cellular oxidation-reduction conditions) may regulate 12-lipoxygenase activity. The potent inhibitory effect of the epithelial cytosolic fraction on microsomal 12-lipoxygenase activity and the lack of complete reversal of the inhibitory effect using GSH-depleting agents may even suggest the presence of additional regulatory controls over the microsomal 12-lipoxygenase. Lipoxynase protein-protein interaction in the form of membrane-bound proteins that combine with the 5-lipoxygenase to generate a complex that catalyzes leukotriene formation has been described (39), but there is no direct evidence as yet for the expression of an analogous protein that alters 12-lipoxygenase activity. Nonetheless, the present results suggest that the 12-lipoxygenase expressed in cultured epithelial cells may contain distinct structural features which allow for microsomal membrane localization and selective sensitivity to by cellular oxidation-reduction conditions. The capacity of endogenous GSH to inhibit microsomal-type 12-lipoxygenase may offer a distinct mechanism for the resistance of epithelial barrier cells to oxidative damage. Alternatively, the ability of lipid hydroperoxide to activate microsomal-type 12-lipoxygenase may offer a means to directly augment the peroxidative membrane damage that takes place in response to the release of hydroperoxides during inflammation.

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