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Airway epithelial cells synthesize the lipid mediator 5-oxo-ETE in response to oxidative stress

Karl-Rudolf Erle^{mann}*,[¶], Chantal Cossette*, Sylvie Gravel*, Alain Lesimple[†], Gue-Jae Lee[‡], Goutam Saha[‡],[§], Joshua Rokach[‡], and William S. Powell*

* Meakins-Christie Laboratories, Department of Medicine, McGill University, 3626 St. Urbain Street, Montreal, Quebec, Canada H2X 2P2

† Mass Spectrometry Unit, McGill University, 740 Dr Penfield, Montreal, QC, Canada, H3A 1A4

‡ Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, Florida 32901-6982, USA

SUMMARY

5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a potent eosinophil chemoattractant that is synthesized from the 5-lipoxygenase product 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by the NADP⁺-dependent enzyme 5-hydroxyeicosanoid dehydrogenase (5-HEDH), previously reported only in inflammatory cells. Because of their critical location at the interface of the lung with the external environment, we sought to determine whether epithelial cells could also synthesize this substance. We found that HEp-2, T84, A549, and BEAS-2B cells all synthesize 5-oxo-ETE in amounts comparable to leukocytes. The epithelial dehydrogenase is localized in the microsomal fraction, requires NADP⁺, and is selective for the *S*-isomer of 5-HETE, suggesting that it is identical to leukocyte 5-HEDH. Normal human bronchial epithelial cells have an even greater capacity to synthesize 5-oxo-ETE. H₂O₂ dramatically stimulates its synthesis in association with increased levels of intracellular GSSG and NADP⁺. These responses were all blocked by removal of GSH/GSSG with N-ethylmaleimide, suggesting that H₂O₂ stimulates 5-oxo-ETE synthesis by raising NADP⁺ levels through activation of the GSH redox cycle. Airway smooth muscle cells can also synthesize 5-oxo-ETE, but to a lesser extent. These results suggest that epithelial cells may be a major source of 5-oxo-ETE under conditions of oxidative stress, which may contribute to eosinophil infiltration in allergic diseases.

Keywords

5-HETE; 5-Hydroxyeicosanoid dehydrogenase; 5-Lipoxygenase products; 5-Oxo-ETE; Airway epithelial cells; Airway smooth muscle cells; Eicosanoids; Glutathione; Hydrogen peroxide; Inflammation; NADP⁺

Corresponding author: William S. Powell, Meakins-Christie Laboratories, McGill University, 3626 St. Urbain Street, Montreal, Quebec, Canada H2X 2P2, Telephone: 514-398-3864 ext. 094071, FAX: 514-398-7483, E-Mail: William.Powell@McGill.ca.

[¶] Present address: Charles River Laboratories Preclinical Services Montreal Inc., 22022 Autoroute Transcanadienne, Senneville (Montreal), Quebec, Canada H9X 3R3

[§] Present address: Goutam Saha, Chembiotech Research International, Block BN, Sector V Salt Lake City, Kolkata 700 091, India

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INTRODUCTION

5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-EETE), an arachidonic acid metabolite derived from the 5-lipoxygenase (5-LO) pathway, is a potent chemoattractant for human eosinophils [1–3] and neutrophils [4,5], and stimulates a wide range of responses in these cells [6]. It is particularly active on human eosinophils, inducing a stronger chemotactic response in these cells than any other lipid mediator [1]. It also activates monocytes [7] and basophils [8–10], but induces a more limited range of responses in these cells. In addition to its effects on eosinophil migration, 5-oxo-EETE can also promote the survival of these cells through its stimulatory effects on GM-CSF release from monocytes [11]. When administered *in vivo* it elicits the infiltration of eosinophils into rat lungs [12] and both eosinophils and neutrophils into human skin [13]. 5-Oxo-EETE acts through a G_i-coupled receptor that is most highly expressed in eosinophils > neutrophils > macrophages [14,15].

5-Oxo-EETE is synthesized by the oxidation of 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) [16]. Although its sequence has not yet been determined, this enzyme is known to be highly specific for substrates containing a 5S-hydroxyl group followed by a 6-trans double bond, with 5S-HETE being the preferred substrate [16]. In contrast, little or no metabolism is observed for 5R-HETE, leukotriene B₄ (LTB₄), which has a 6-cis double bond), or positional isomers of 5-HETE such as 12-HETE and 15-HETE. 5-HEDH is found in various inflammatory cells, including granulocytes, mononuclear cells, and platelets [6], but has not previously been demonstrated in any structural cells. The synthesis of 5-oxo-EETE also requires NADP⁺ [17], the intracellular levels of which are normally quite low [18]. Activation of the respiratory burst in phagocytic cells [17] and exposure of mononuclear cells and platelets to oxidative stress [19] results in enhanced synthesis of 5-oxo-EETE. Indirect evidence suggests that this may be due to increased intracellular levels of NADP⁺, although cofactor levels were not measured directly.

Although 5-oxo-EETE has been detected in human lung tissue by mass spectrometry [20], the site of its synthesis in the lung was not identified. Increased amounts of 5-oxo-EETE were found in lung samples from subjects with pulmonary hypertension, a condition that is associated with increased oxidative stress [20]. Allergic inflammation in the lung is also associated with oxidative stress, which particularly affects epithelial cells and macrophages [21]. Airway epithelial cells are at the interface of the body with the external environment, and synthesize a variety of peptide chemokines that appear to be involved in inflammatory responses [22]. However, it is not known whether these cells, or any other structural cells, have the ability to synthesize the potent lipid eosinophil chemoattractant 5-oxo-EETE. The objective of the current study was to determine whether epithelial cells could be an important site for the synthesis of 5-oxo-EETE, and, if so, whether this is affected by oxidative stress.

MATERIALS AND METHODS

Materials

5-HETE [23] and 5-oxo-EETE [24] were prepared by chemical synthesis as described previously. The following were purchased from Sigma-Aldrich Corp., St. Louis, MO: phenazine methosulfate (PMS), N-ethylmaleimide (NEM), tert-butyl hydroperoxide (tBuOOH), H₂O₂ (30% solution), diamide, o-phthalaldehyde, acetophenone, formic acid, NAD⁺, Deamino-NAD⁺, tetrabutyl ammonium hydroxide (TBAH), and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Polyethylene glycol 600 diacid was purchased from Fluka (Oakville, ON, Canada). Dimethyl sulfoxide (DMSO) and sodium azide was purchased from Fisher Scientific (Nepean, Ontario, Canada), whereas NADP⁺ was from Roche Diagnostics.

Epithelial and smooth muscle cells

Epithelial cells lines (A549, BEAS-2B, T84, and HEp-2) were obtained from the American Type Culture Collection (Table 1) and cultured in 10% FBS in DMEM/F12 medium containing L-glutamine (2 mM), sodium bicarbonate (2.5 g/L), penicillin (100U/ml), and streptomycin (100 µg/ml). Cells were sub-cultured before reaching confluence and used for up to 20 passages. Normal human bronchial epithelial (NHBE) cells and human bronchial smooth muscle cells (BSMC) were obtained from Cambrex (San Diego, CA, USA) and cultured according to the supplier's directions. Cells were seeded at a density of 3500 cell/cm² and grown to 80% confluency with the medium being changed every 2 days. NHBE and BSMC cells were received at passages 1 and 3, respectively, and used until passages 8 and 10, respectively. Experiments were performed on cells cultured in 6-well plates.

Preparation of subcellular fractions from A549 cells

The cells were washed by centrifugation, resuspended in 20 ml PBS supplemented with 1 mM PMSF, and disrupted by sonication at a setting of 40 cycles/s (model 4710 ultrasonic homogenizer, Sonics and Materials, Danbury, DC) on ice for 5 × 6 s with 30 s intervals for cooling. The homogenate was successively centrifuged at 4 °C at 1,500 × g for 10 min, 10,000 × g for 10 min, and 150,000 × g for 80 min and the final pellet was resuspended in PBS. Protein was measured with the Bio-Rad DC (detergent compatible) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to the indicated concentrations.

Measurement of oxo-eicosanoids by precolumn extraction-reversed-phase high performance liquid chromatography (RP-HPLC)

Incubations were terminated by the addition of methanol (0.65 ml) and cooling to 0 °C. The concentration of methanol in each sample was then adjusted to 30% by addition of water and 13-HODE (100 ng; internal standard). HETE metabolites were analyzed by precolumn extraction/RP-HPLC [25] using a modified Waters Alliance system (Waters Associates, Milford, MA). The stationary phase was a C₁₈ column (150 x 3.9 mm) of octadecylsilyl (ODS)-silica (4 µm particle size Novapak column, Waters). The mobile phase was a linear gradient composed of solvents A (water/acetic acid (100:0.02)) and B (acetonitrile/acetic acid (100/0.02)) as follows: 0 min: 65% B; 6 min, 82% B; 8 min, 82%B. The retention times were as follows: 13-HODE (5.1 min), 5-HETE (5.9 min), and 5-oxo-EETE (6.7 min). All solvents were of HPLC grade. Products were quantitated by comparing the areas of their peaks of UV absorbance at their λ_{max} with that of the internal standard, 13-HODE, and were corrected for differences in extinction coefficients, which were as follows: 13-HODE, 23,000; 5-HETE, 27,000; 5-oxo-EETE, 22,000. The identities of the products measured were confirmed by examination of their complete UV spectra (cf. Fig. 4C, below). We have previously shown that 5-oxo-EETE is well separated from other oxo-EETEs and HETEs by RP-HPLC [26]

Identification of epithelial cell-derived 5-oxo-EETE by Fourier-transform mass spectrometry mass spectrometry (FTMS)

A549 cells were cultured to 80% confluence, trypsinized, and resuspended in PBS at a density of 10⁶ cells/ml. The cells (12 ml) were then incubated for 1 h at 37 °C with 5-HETE (4 µM) in the presence of H₂O₂ (100 µM). The incubation was terminated by addition of MeOH to give a final concentration of 30%, followed by centrifugation. The supernatant was subjected to solid-phase extraction on a cartridge containing octadecylsilyl silica (C₁₈ Sep-Pak, Waters) as previously described [27], except that the sample was loaded in 30% MeOH at neutral pH. The products were purified by RP-HPLC on a Spherisorb ODS-2 column (250 x 4.6 mm; 5 µm particle size; Phenomenex, Torrance, CA) using 50% aqueous acetonitrile containing 0.02% acetic acid as the mobile phase. 5-Oxo-EETE, detected at 280 nm, had a retention time of 33 min.

The identity of 5-oxo-ETE was confirmed by direct infusion electrospray ionization in negative ion mode, using an IonSpec 7.0 tesla Fourier transform mass spectrometer (Lake Forest, CA, USA) calibrated with polyethylene glycol 600 diacid. A Z-spray source from Waters Corporation (Milford, MA, USA) was used with a capillary voltage of 2800 V, a cone voltage of 30V and a sample flow rate of 1–3 mL/hr. The solvent used was a 2:1 mixture of 90% MeOH containing 25 mM of ammonium acetate and CH₂Cl₂. The elemental composition of 5-oxo-ETE was confirmed in MS mode and the structure through MS². MS² experiments were performed after isolating the monoisotopic parent ion using 500 ms of Sustained Off-Resonance (1kHz) Irradiation Collision Induced Dissociation and a 90 ms nitrogen gas pulse. The obtained scan was internally calibrated using the exact mass of the parent ion.

Measurement of GSH and GSSG

GSH and GSSG were measured by RP-HPLC using a post-column derivatization procedure modified from the literature [28]. Cells cultured on 6-well plates were incubated with H₂O₂ (100 μM) and the medium rapidly removed after various times. Incubations were terminated by addition of 200mM phosphoric acid (1 ml) containing 12mM CHAPS at 0 °C. After vigorous mixing with a pipette, the samples were transferred to tubes, which were stored at –80 °C until analysis. GSH and GSSG levels were determined in 50 μl aliquots by RP-HPLC using a gradient prepared from 0.05% trifluoroacetic acid (TFA) in water (solvent C) and 0.05% TFA in acetonitrile (solvent D) as follows: 0 min, 0% D; 10 min, 15% D. The flow rate was 1 ml/min and the stationary phase was a column (150 x 4.6 mm) of Ultracarb ODS (31% carbon loading; 5 μm particle size; 150 x 4.6 mm; Phenomenex, Torrance, CA). The eluate from the column was mixed with o-phthalaldehyde (370 μM) in 0.2 M tribasic sodium phosphate, pH 12, which was pumped into a T-fitting using an auxiliary pump (Waters Reagent Manager). The mixture then passed through a loop of PEEK tubing (6 m x 0.5 mm, i.d.; volume, 1.2 ml) that was placed in a water bath at 70 °C. Under these conditions both GSH and GSSG are converted to a fluorescent isoindole adduct, which is measured using excitation and emission wavelengths of 336 and 420 nm, respectively. Prior to introduction into the fluorescence detector (Waters model 2475 Multi wavelength Fluorescence Detector), the mixture was cooled in a small ice-water bath and passed through a filter containing an OptiSolv 0.2 μm frit from (Optimize Technologies). The amounts of GSH and GSSG were determined from a standard curve using the authentic compounds as external standards.

Analysis of intracellular NADP⁺

NADP⁺ was measured by RP-HPLC following derivatization with alkaline acetophenone to give a fluorescent naphthyridine derivative (cf. ref. [29]; full details to be published elsewhere). Briefly, cells in 6-well plates were incubated with H₂O₂ for various times, the medium rapidly removed, and the reactions stopped by addition of an ice-cold solution containing 50 mM acetophenone and 3 N KOH in 50% MeOH. Deamino-NAD⁺ (100 ng in 10 μl PBS) was added as an internal standard. The mixture was kept at 0 °C for 20 min, after which time formic acid (62.5 μl) was added. After a further 5 min at 0 °C, the mixture was extracted with ethyl acetate (2 x 1.5 ml) and the aqueous phase treated with phenazine methosulfate (100 μM). The samples were stored at –80 °C until further analysis (usually within one week). Aliquots (50 μl) were then analyzed by RP-HPLC on an Ultracarb ODS column (see above). The mobile phase consisted of a gradient between solvent E (100 mM citric acid containing 4 mM tetrabutyl ammonium bisulfate (TBAB) and solvent F (acetonitrile) as follows: 0 min, 1% F; 12 min, 25% F; 14.3 min, 25% F). The flow rate was 1.25 ml/min. The NADP⁺-acetophenone adduct was detected using excitation and emission wavelengths of 371 and 438 nm, respectively. The ratios of peaks areas for NADP⁺ and deamino-NAD⁺ were calculated and the amount of NADP⁺ determined from a standard curve.

RESULTS

Epithelial cell lines synthesize 5-oxo-ETE

To determine whether the epithelium is capable of producing 5-oxo-ETE from 5-HETE, we screened four different epithelial cell lines (HEp-2, T84, A549 and BEAS-2B). As 5-oxo-ETE synthesis from 5-HETE appears to be limited by the availability of the 5-HEDH cofactor NADP⁺, the cells were preincubated with PMS, which nonenzymatically converts intracellular NADPH to NADP⁺ [30]. We have previously used this method to give an approximation of the capacity of leukocytes to synthesize 5-oxo-ETE [19]. PMS alone does not oxidize 5-HETE, but requires the presence of 5-HEDH and NADPH [17]. All of the epithelial cell lines investigated converted 5-HETE to substantial amounts of 5-oxo-ETE in the presence of PMS (Fig. 1). The most active were T84 intestinal epithelial cells and A549 airway epithelial cells, both of which produced in excess of 400 pmol 5-oxo-ETE/10⁶ cells, comparable to the amount produced by human monocytes (387 ± 16 pmol/10⁶ cells) under identical conditions (data not shown).

Characterization of epithelial 5-HEDH activity

The 5-HEDH activity in A549 was examined in more detail to determine whether it is similar to the myeloid enzyme (which has not yet been cloned). To examine the substrate specificity, cofactor requirements, and subcellular localization, we incubated cytosol, granule, and microsomal fractions with 5-HETE, 12-HETE, and 15-HETE in the presence of NADP⁺ or NAD⁺. The dehydrogenase-derived products (i.e. 5-oxo-ETE, 12-oxo-ETE, and 15-oxo-ETE, respectively) formed from each of the above substrates were then measured by RP-HPLC. As shown in Fig. 2A, microsomal fractions from A549 cells displayed by far the greatest degree of 5-HEDH activity in the presence of NADP⁺ (1.03 ± 0.32 pmol 5-oxo-ETE/min/μg protein). Somewhat lower activity was observed in the granule fraction (Fig. 2B), whereas the cytosol possessed very little activity (Fig. 2C). The A549 cell dehydrogenase was highly selective for 5S-HETE, and displayed very little activity towards 5R-HETE (0.04 ± 0.01 pmol/min/μg protein; Fig. 2A, inset). The lack of metabolism of 5R-HETE provides further evidence that the oxidation of 5S-HETE to 5-oxo-ETE is an enzyme-catalyzed process. In contrast to 5-HETE, only very small amounts of 12-HETE and 15-HETE were oxidized by subcellular fractions from A549 cells. Microsomal fractions converted 12-HETE to small amounts of 12-oxo-ETE (0.10 ± 0.07 pmol/min/μg protein), but this reaction was favored by NAD⁺ to a greater extent than NADP⁺. 15-HETE was metabolized to a small extent (0.08 ± 0.03 pmol/min/μg protein) by the cytosolic fraction in the presence of NADP⁺, probably by Type II 15-hydroxyprostaglandin dehydrogenase (15-PGDH).

To determine the K_m and V_{max} values for the A549 cell dehydrogenase, microsomal fractions were incubated with various concentrations of 5-HETE in the presence of NADP⁺. Lineweaver-Burk plots of the data (Fig. 3) gave values of 890 ± 150 nM for the K_m and 1.6 ± 0.5 pmol/min/μg protein for the V_{max}.

Oxidative stress stimulates 5-oxo-ETE synthesis by A549 cells

A549 cells were subjected to oxidative stress by exposure to H₂O₂ and incubated with 5-HETE for 20 min. The products were analyzed by RP-HPLC, using 13-HODE as an internal standard. The conditions employed completely separated the internal standard (t_R, 5.1) from 5-HETE (t_R, 5.9), and 5-oxo-ETE (t_R, 6.7) (Figs. 4A and 4B), which were identified on the bases of their UV spectra (Fig. 4C). The peak corresponding to 5-HETE had a λ_{max} at 235 nm but did not absorb at 280 nm, whereas that for 5-oxo-ETE had a λ_{max} at 280 nm. A549 cells produced only a relatively small amount of 5-oxo-ETE in the absence of H₂O₂ (Fig. 4A), whereas in its presence a much larger amount was formed (Fig. 4B).

To further confirm the identity of 5-oxo-ETE, this compound was isolated by RP-HPLC after incubation of A549 cells with 5-HETE and analyzed by high resolution electrospray ionization-FTMS. In MS mode, an intense ion at m/z 317.21224 ($C_{20}H_{29}O_3^-$ requires 317.21222) was observed as expected for the carboxylate anion of 5-oxo-ETE. Collision-induced dissociation of this ion resulted in a daughter ion spectrum (Fig. 4D) with major ions at m/z 273.22249 ($C_{19}H_{29}O^-$ requires 273.22239; loss of the carboxyl group) and 203.18056 ($C_{15}H_{23}^-$ requires 203.18052; loss of C_1-C_5 due to cleavage adjacent to the 5-oxo group). An ion of low intensity was observed at m/z 113.02440 ($C_5H_5O_3^-$ requires 113.02442; $O=C=CH-CH_2=CH_2-CO_2^-$) corresponding to C_1-C_5 . Additional ions were present with m/z values of 299.20178 ($C_{20}H_{27}O_2^-$ requires 299.20165) and 255.21192 ($C_{19}H_{27}^-$ requires 255.21182), presumably due to loss of H_2O from the ions at m/z 317 and 273, respectively. The $[M-H]^-$ ion at m/z 317 and the associated ions at m/z 299 and 273 due to loss of H_2O and CO_2 , respectively, indicate that the product with a t_R of 6.7 min (Fig. 4A and 4B) is an oxo-ETE, whereas the intense ion at m/z 203 (cf. refs [31,32]) as well as the one at m/z 113 establish that the oxo group is in the 5-position.

The time courses for the effects of H_2O_2 and tBuOOH (100 μM) on 5-oxo-ETE formation are shown in Fig. 5A. Both of these agents strongly stimulated 5-oxo-ETE synthesis in a time-dependent manner. The effect of H_2O_2 was maximal at 20 min, and then declined, whereas the response to tBuOOH persisted up to at least 40 min. To determine whether the response to H_2O_2 was limited by its breakdown by catalase, A549 cells were preincubated for 30 min with sodium azide (1 mM) prior to its addition. This resulted in considerably enhanced 5-oxo-ETE synthesis at the later time points (Fig. 5A). The concentration-response relationships for the effects of H_2O_2 and tBuOOH on 5-oxo-ETE synthesis, measured after 20 min, are shown in Fig. 5B. H_2O_2 and tBuOOH had similar EC_{50} values in stimulating 5-oxo-ETE formation (35 ± 6 and 31 ± 6 μM , respectively). The maximal response for tBuOOH appeared to be a little higher, but this difference was not significant. Addition of diamide (250 μM), which non-enzymatically oxidizes intracellular GSH to GSSG, had an effect almost identical to H_2O_2 (Fig. 5C).

Normal human bronchial epithelial cells synthesize 5-oxo-ETE in response to oxidative stress

The above results with cell lines, suggested that epithelial cells could be an important source of the inflammatory mediator 5-oxo-ETE in the lung. To ensure that this phenomenon is not restricted to cell lines, it was important to determine whether normal airway epithelial cells also had the ability to synthesize this substance. As shown in Fig. 6, normal human bronchial epithelial (NHBE) cells are very active in synthesizing 5-oxo-ETE in response to H_2O_2 (100 μM). The effect of H_2O_2 was much more prolonged compared to A549 cells, the formation of 5-oxo-ETE being nearly linear up to 40 min. The response to H_2O_2 was completely blocked by preincubation with the thiol alkylating agent N-ethylmaleimide (NEM; 100 μM) for 10 min. In contrast to A549 cells, addition of azide had no effect on 5-oxo-ETE formation by NHBE cells at any time point up to 40 min (data not shown). tBuOOH also stimulated 5-oxo-ETE synthesis, with a time course virtually superimposable on that for H_2O_2 (data not shown). Diamide also had an effect very similar to that of H_2O_2 (data not shown).

Effects of H_2O_2 on GSH and GSSG levels in NBHE cells

The strong stimulatory and inhibitory effects of diamide and NEM, respectively, on H_2O_2 -induced 5-oxo-ETE synthesis suggested that this response was dependent on the glutathione redox cycle. To provide more evidence for this, we measured GSH and GSSG by HPLC using post-column derivatization with o-phthalaldehyde at alkaline pH. With this procedure, GSH and GSSG are first separated by HPLC and are then each converted to a highly fluorescent isoindole derivative that can readily be detected using a fluorescence detector. In unstimulated

cells, nearly all of the intracellular glutathione was in the reduced form (Fig. 7A), with only very low levels of GSSG being detected. This relationship was nearly completely reversed by addition of H_2O_2 (100 μM), which dramatically increased GSSG levels and reduced GSH levels after 3 min (Fig. 7B). To ensure that H_2O_2 did not oxidize GSH during the work-up procedure, it was added to cells immediately after addition of the stopping solution (H_3PO_4 /CHAPS). This did not result in the formation of GSSG, as the measured levels of GSH and GSSG were the same as the levels in control cells not treated with H_2O_2 (data not shown).

The time course for the effect of H_2O_2 on glutathione levels is shown in Fig. 7C. GSH fell precipitously from 73.5 ± 9.7 to 13.2 ± 3.0 nmol/ 10^6 cells one min after the addition of H_2O_2 , whereas GSSG levels increased from 0.2 ± 0.1 to 32.1 ± 3.9 nmol/ 10^6 cells during the same period (note that in Fig. 7C, glutathione disulfide is shown as $\frac{1}{2}$ GSSG). The effects of H_2O_2 were maximal at 3 min, with GSSG levels gradually declining thereafter. However, even after 20 min, $\frac{2}{3}$ of the total glutathione still existed in the form of the disulfide. After pretreatment of NHBE cells with NEM for 10 min both GSH and GSSG were undetectable (data not shown).

Elevation of NADP^+ levels in NHBE cells in response to H_2O_2

The principal enzyme responsible for maintaining glutathione in the reduced state in cells is glutathione reductase, which reduces GSSG to GSH at the expense of oxidation of NADPH to NADP^+ [33]. We therefore measured the intracellular levels of NADP^+ following treatment of NHBE cells with H_2O_2 by HPLC after its conversion to a fluorescent 1,6-naphthylridine derivative by treatment with acetophenone. Unstimulated cells contained only low levels of NADP^+ (53 ± 11 pmol/ 10^6 cells), which dramatically increased to 466 ± 151 pmol/ 10^6 cells 1 min following addition of H_2O_2 , and further increased to 887 ± 147 pmol/ 10^6 cells after 20 min (Fig. 8). The increase in NADP^+ levels was completely blocked by pretreatment of NHBE cells with NEM.

5-Oxo-ETE synthesis by airway smooth muscle cells

To determine whether airway smooth muscle cells also have the ability to synthesize 5-oxo-ETE, human bronchial smooth muscle cells (BSMC) were incubated with 5-HETE for various times in the presence and absence of H_2O_2 . As shown in Fig. 9A, these cells also possess 5-HEDH activity. As with epithelial cells, 5-oxo-ETE formation is stimulated by H_2O_2 , but the maximal amount formed is considerably less and the rate of formation diminishes with time, in contrast to epithelial cells, with which its formation is almost linear up to 40 min. GSSG levels increase dramatically in BSMC following addition of H_2O_2 , reaching a maximum after 1 min and then declining to 20% of the maximal level by 20 min (Fig. 9B). Similarly, NADP^+ levels increased rapidly in response to H_2O_2 , reaching maximal levels by 1 min and then declining (Fig. 9C). Pretreatment with NEM completely depleted the cells of GSH and GSSG (Fig. 9B) and blocked the effects of H_2O_2 on 5-oxo-ETE synthesis and NADP^+ levels.

DISCUSSION

The present study demonstrates that epithelial cells from a variety of sources express high levels of 5-HEDH. We focused primarily on pulmonary epithelial cells because of the potential importance of 5-oxo-ETE as an eosinophil chemoattractant in asthma. However, we also found that 5-HEDH activity is expressed in T84 intestinal epithelial cells, suggesting that it may be present in epithelial cells from a variety of tissues. Furthermore, the expression of this enzyme is not limited to cell lines, as it is also present in normal human bronchial epithelial cells. Thus 5-HEDH is present on a wider variety of cells than first thought, as it was initially identified only in leukocytes and platelets. Epithelial cells also express another enzyme in the 5-LO pathway, LTA_4 hydrolase, which is required for the production of LTB_4 . [34]. This compound

is a potent neutrophil chemoattractant [35] but, unlike 5-oxo-ETE, has little effect on the migration of human eosinophils [1,36]. Although normal epithelial cells appear to possess little to no 5-LO activity, this activity can be induced following exposure to pathogens [37]. Alternatively, in pathological situations 5-HETE is likely to be provided by infiltrating inflammatory cells or by activated resident macrophages, and converted to 5-oxo-ETE by epithelial cells (transcellular biosynthesis).

To determine whether the formation of 5-oxo-ETE in epithelial cells is likely to be due to the same enzyme that is responsible for this reaction in leukocytes, we examined the properties of the A549 cell dehydrogenase in more detail. Like the leukocyte enzyme, epithelial 5-HEDH displays high selectivity for 5-HETE, requiring a substrate with a 5-hydroxyl group in the S configuration. Furthermore, as with leukocyte 5-HEDH, the epithelial enzyme is expressed most highly in the microsomal fraction and is selective for the cofactor NADP⁺ over NAD⁺. The level of 5-HEDH activity in A549 cell microsomes ($V_{\max}$, 1.6 ± 0.5 pmol/min/ μ g protein) is about 3 times higher than that which we previously found in human neutrophil microsomes ($V_{\max}$, 0.5 pmol/min/ μ g protein).

12-HETE was also oxidized by A549 cell microsomes, but only to a very small extent compared to 5-HETE. Moreover, the preferred cofactor appears to be NAD⁺ rather than NADP⁺. This suggests that microsomal 12-hydroxyeicosanoid dehydrogenase (12-HEDH) may be responsible for this activity, as it prefers NAD⁺ as cofactor [38]. Products formed by the 12-HEDH pathway have previously been identified in human lung [39]. A small amount of oxidation of 15-HETE was also observed, but this could not be due to 5-HEDH, as it occurred to the greatest extent in the cytosolic fraction. This activity may be attributable to type II 15-PGDH, which prefers NADP⁺ as cofactor, but can also use NAD⁺. This is a relatively nonspecific enzyme that also reduces a variety of carbonyl compounds in the presence of NADPH [40]. We also found that PGF_{2 α} is metabolized to a similar extent by this fraction in the presence of NADP⁺ (data not shown).

As in leukocytes, the basal synthesis of 5-oxo-ETE in unstimulated epithelial cells is relatively low. In leukocytes this appears to be due to the low intracellular levels of NADP⁺, as 5-oxo-ETE synthesis is stimulated by treatment with agents that can raise the levels of this cofactor. With mononuclear cells and platelets, this can be accomplished by exposure to oxidative stress. In contrast, 5-oxo-ETE synthesis in neutrophils is not regulated by oxidative stress, but is instead stimulated by activation of NADPH oxidase [6]. In the present study we initially investigated the capacity of different epithelial cell lines to synthesize 5-oxo-ETE by treating these cells with PMS, which nonenzymatically converts intracellular NADPH to NADP⁺ [30]. To learn more about the physiological regulation of 5-oxo-ETE synthesis by these cells we investigated the effects of oxidative stress on this process in A549 cells. We found that both H₂O₂ and tBuOOH are potent stimulators of the formation of this substance, with EC₅₀ values of about 30 μ M. The response to H₂O₂ does not persist at longer time points due to its metabolism by catalase, as its effect can be prolonged by addition of azide, an inhibitor of this enzyme. The effect of H₂O₂ was mimicked by diamide, which nonenzymatically oxidizes intracellular GSH to GSSG, suggesting the involvement of the GSH redox cycle (Fig. 10).

The ability of epithelial cells to synthesize 5-oxo-ETE was not restricted to tumor cell lines, but was also observed in normal human bronchial epithelial cells, and was strongly stimulated by H₂O₂, suggesting that oxidative stress is a regulator of 5-oxo-ETE synthesis by epithelial cells in general. The effect of H₂O₂ persisted much longer in NHBE cells than in A549 cells, and was not enhanced by addition of azide. The shorter duration of action of H₂O₂ in A549 cells may therefore be due to higher expression of catalase activity compared to NHBE cells.

To determine whether other structural cells in the lung can synthesize this lipid mediator, we examined normal human bronchial smooth muscle cells and found that they also have the ability to produce 5-oxo-ETE, although to a lesser extent than in epithelial cells (ca. 1 nmol vs 2.5 nmol 5-oxo-ETE/10⁶ cells after treatment with H₂O₂ for 40 min). Thus both cell types could contribute to 5-oxo-ETE synthesis following exposure of the lung to oxidative stress.

The effect of H₂O₂ on 5-oxo-ETE synthesis by airway epithelial cells appears to be mediated by the GSH redox cycle, as it was mimicked by diamide and blocked by the thiol alkylating agent NEM (Fig. 10). Furthermore, H₂O₂ dramatically increased the intracellular levels of GSSG and NADP⁺, both of which were measured directly by HPLC. Three min after stimulation of NHBE cells with H₂O₂, GSSG levels amounted to 96% of total intracellular glutathione, compared to only 0.4% in unstimulated cells. GSSG declined somewhat thereafter, but still remained very high, and after 20 min amounted to 66% of total glutathione. These high levels of GSSG would likely have saturated glutathione reductase, thus providing a constant supply of NADP⁺ for 5-oxo-ETE synthesis.

The lesser ability of BSMC to produce 5-oxo-ETE compared to NHBE cells could be due either to lower expression of 5-HEDH or to differences in GSSG and NADP⁺ levels. Although H₂O₂ induced rapid increases in both GSSG and NADP⁺ in BSMC, the maximal levels reached were about 6 times higher in NHBE cells than BSMC for GSSG and about 4 times higher for NADP⁺. Moreover, the level of NADP⁺ reached a maximum after 1 min in BSMC, and then declined, in contrast to NHBE cells, in which it continued to rise up to 20 min. This might explain the nearly linear relationship of 5-oxo-ETE synthesis with time for NHBE cells.

As well as being a source of lipid mediators, epithelial cells produce a variety of chemokine peptides, including IL-8 [41], MCP-1 [42], RANTES [43], and eotaxin [44]. Airway smooth muscle cells are also a source of various chemokines [22]. Interestingly, synergistic interactions between 5-oxo-ETE and some of the above chemokines have been observed. For example, low concentrations of eotaxin and RANTES enhance the chemotactic response of eosinophils to 5-oxo-ETE [45]. Furthermore, 5-oxo-ETE, in addition to inducing monocyte migration on its own, increases the responsiveness of these cells to MCP-1 [7]. Thus epithelial cells, which form the interface with the external environment, can produce a variety of both lipid and peptide chemoattractants that likely combine to induce the infiltration of inflammatory cells in response to invading microorganisms and noxious stimuli.

The ability of intestinal epithelial cells to synthesize 5-oxo-ETE may also have important implications for the regulation of eosinophils in the gut, as these cells are abundant in this tissue [46]. Moreover, 5-oxo-ETE has been shown to induce volume reduction in guinea pig intestinal crypt epithelial cells, a response that was dependent on activation of chloride channels [47].

In conclusion, the high levels of 5-HEDH activity in epithelial cells, and the ability of these cells to synthesize large amounts of 5-oxo-ETE following stimulation by oxidative stress, suggests that these cells may be a major site for the synthesis of this lipid mediator in tissues such as the lung. Epithelial cells are particularly susceptible to oxidative stress-induced changes associated with inflammation, as they have been shown to accumulate high levels of isoprostanes following allergen challenge of sensitized mice [21]. In this environment, these cells could produce substantial amounts of 5-oxo-ETE, which could contribute to the infiltration of eosinophils into the lung [12], as well as increase the survival of these cells through its stimulatory effect on GM-CSF secretion [11], resulting in prolongation of the inflammatory response.

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ABBREVIATIONS

5-HEDH

5-hydroxyeicosanoid dehydrogenase

5-HETE

5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid

5-LO

5-lipoxygenase

5-oxo-EETE

5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid

5R-HETE

5R-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid

12-HEDH

12-hydroxyeicosanoid dehydrogenase

12-HETE

12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid

12-oxo-ETE	12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid
13-HODE	13S-hydroxy-9Z,11E-octadecadienoic acid
15-HETE	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
15-PGDH	15-hydroxyprostaglandin dehydrogenase
BSMC	human bronchial smooth muscle cells
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
FTMS	Fourier-transform mass spectrometry
GM-CSF	Granulocyte macrophage-colony stimulating factor
IL-8	interleukin-8
LT	leukotriene
MCP-1	monocyte chemoattractant protein-1
NEM	N-ethylmaleimide
NHBE	normal human bronchial epithelial
ODS	octadecylsilyl
PBS	phosphate-buffered saline
PMS	phenazine methosulfate
PMSF	phenylmethanesulfonyl fluoride
RANTES	regulated upon activation in normal T cells expressed and secreted
RP-HPLC	reversed-phase high performance liquid chromatography
TBAH	tetrabutyl ammonium hydroxide

tBuOOH

tert-butyl hydroperoxide

TFA

trifluoroacetic acid

Fig. 1. Synthesis of 5-oxo-EETE by epithelial cell lines

Epithelial cells were cultured to confluence in 6-well plates. Cells were washed twice with PBS+ and then preincubated for 6 min with 100 μ M PMS. The cells were then incubated for an additional 20 min with 4 μ M 5-HETE. Cells in adjoining wells were detached with trypsin and counted. Values are means $\pm$ SE ($n = 4$, except for T84 and HEP-2 cells ($n=2$)).

Fig. 2. Substrate selectivity, cofactor requirements, and subcellular localization of A549 cell eicosanoid dehydrogenase activity

Subcellular fractions from A549 cells, prepared as described in Material and Methods, were incubated with 4 μ M 5-HETE, 12-HETE, or 15-HETE for 20 min in the presence of either 100 μ M NADP⁺ (filled bars) or 100 μ M NAD⁺ (open bars). The values are the amounts of oxo metabolites formed by (A) microsomes (50 μ g protein/ml), (B) granules (150 μ g protein/ml), and (C) cytosol (400–500 μ g protein/ml) ($n \geq 3$). The inset shows amounts of 5-oxo-EETE formed from 5*S*-HETE and 5*R*-HETE ($n=2$).

Fig. 3. Lineweaver-Burk plot for formation of 5-oxo-EETE by A549 cell microsomes

A microsomal fraction (50 μg protein in 1 ml) derived from A549 cells was incubated with various amounts of 5-HETE in the presence of 100 μM NADP⁺ for 5 min. The data shown are from a one experiment out of a total of 5 separate experiments, all with similar results.

Fig. 4.

Analysis of H_2O_2 -induced 5-oxo-EETE formation by RP-HPLC. A549 cells were incubated for 20 min with 5-HETE ($4 \mu\text{M}$) in the presence of either vehicle (**A**) or $100 \mu\text{M}$ H_2O_2 (**B**) and the products separated by RP-HPLC as described in Materials and Methods. Panel **C** shows the UV spectra of the peaks with retention times of 5.9 min (5-HETE, λ_{max} , 235 nm) and 6.7 min (5-oxo-EETE, λ_{max} , 280 nm) in the chromatogram shown in panel B. 13h, 13-HODE; 5h, 5-HETE; 5oETE, 5-oxo-EETE. Panel **D** shows the daughter ion spectrum obtained following collision-induced dissociation of the M-1 ion from the 5-HETE metabolite with λ_{max} of 280 nm (panel C) and a t_R of 6.7 min (i.e. 5-oxo-EETE; panel B). The product was purified by RP-HPLC and analyzed by FTMS as described in Materials and Methods.

Fig. 5.

Effects of oxidative stress on 5-oxo-EETE formation by A549 cells. **A:** Time courses for the formation of 5-oxo-EETE from 5-HETE (4 μ M) by A549 cells in the presence of vehicle ($\circ$), 100 μ M H₂O₂ (Δ), or 100 μ M t-BuOOH 100 μ M) (tBu; $\bullet$). Cells were initially preincubated for 30 min at 37 $^{\circ}$ C with either vehicle ($\circ$, Δ , $\bullet$) or 1 mM sodium azide (Az) followed by 100 μ M H₂O₂ ($\blacktriangle$). Values are means $\pm$ SE (n = 4). **B:** Concentration-response curves for the effects of H₂O₂ (Δ) and tBuOOH ($\bullet$) on 5-oxo-EETE formation by A549 cells. Confluent cells were incubated with 5-HETE (4 μ M) for 20 min (n = 4). **C:** Effect of diamide on 5-oxo-EETE formation. A549 cells were incubated with 5-HETE (4 μ M) for 20 min at 37 $^{\circ}$ C in the presence of either diamide (250 μ M) or H₂O₂ (100 μ M) (n = 3).

Fig. 6. Stimulatory effect of H₂O₂ on 5-oxo-EETE formation by NBHE cells. NBHE cells were preincubated for 10 min at 37 °C in the presence of vehicle (●, ○; n = 5) or 100 μM NEM (▲; n = 3), and then incubated with 4 μM 5-HETE at 37 °C in the presence of either vehicle (○) or 100 μM H₂O₂ (●, ▲). After different times 5-oxo-EETE was measured by RP-HPLC. All values are means ± SE.

Fig. 7.

Effect of H₂O₂ on the intracellular levels of GSH and GSSG in NHBE cells. Chromatographic profiles of GSH and GSSG from (A) untreated cells and (B) cells treated with H₂O₂ for 3 min at 37 °C. The products were separated by RP-HPLC and converted to fluorescent products by post-column derivatization as described in Materials and Methods. The retention times of GSH and GSSG were identical to those of authentic standards run separately (not shown). C: Time courses for the effects of 100 μM H₂O₂ on the levels of GSH (○) and GSSG (●) in NHBE cells. The values are means ± SE (n = 4).

Fig. 8.

Effect of H₂O₂ on intracellular NADP⁺ levels in NHBE cells. NHBE cells were preincubated for 10 min at 37 °C in the presence of either vehicle (●) or 100 μM NEM (Δ), followed by incubation with 100 μM H₂O₂ for various times. NADP⁺ levels were then measured by RP-HPLC following conversion to a fluorescent naphthyridine derivative as described in Materials and Methods. The values for H₂O₂ alone are means ± SE (n = 5) and for H₂O₂ in the presence of NEM are means ± range (n = 2).

Fig. 9.

Effect of H₂O₂ on 5-oxo-EETE synthesis and glutathione and NADP⁺ levels in BSMC. Cells were preincubated for 10 min at 37 °C in the presence of vehicle (●, ○) or 100 μM NEM (▲), and then incubated with 4 μM 5-HETE at 37 °C in the presence of either vehicle (○) or 100 μM H₂O₂ (●, ▲). After different times 5-oxo-EETE was measured by RP-HPLC (n = 4). **B:** Time dependent changes in GSSG levels in BSMC following treatment with H₂O₂. Cells were preincubated for 10 min with either vehicle (●; n = 6) or 100 μM NEM (Δ; n = 4) and then incubated for various times with 100 μM H₂O₂. **Inset:** GSH levels in BSMC treated with 100 μM H₂O₂ following preincubation for 10 min with either vehicle (○) or 100 μM NEM (▲). **C:** Effects of 100 μM H₂O₂ on intracellular NADP⁺ levels in NHBE cells pretreated for 10 min at 37 °C with either vehicle (●; n = 6) or 100 μM NEM (Δ; n = 5). All values are means ± SE.

Fig. 10.

Scheme showing the regulation of 5-oxo-EETE synthesis in airway epithelial cells. Metabolism of H_2O_2 and tBuOOH by glutathione peroxidase (GPerox) results in the formation of GSSG, which is reduced back to GSH by glutathione reductase (GRed), generating $NADP^+$, which serves as a cofactor for 5-HEDH. This process can be blocked by NEM, which alkylates GSH, and mimicked by diamide (DA), which oxidizes GSH, and PMS, which oxidizes $NADPH$. In A549 cells the action of H_2O_2 is limited by metabolism by catalase, which can be blocked by azide.

Table 1

Epithelial cells used in this study

Cell type	Origin	Source
HEp-2	HeLa cell contaminant of epidermoid carcinoma cells	ATCC*
T84	Cells obtained following metastasis of a colon carcinoma to the lung	ATCC
A549	Lung carcinoma cells capable of surfactant synthesis	ATCC
BEAS-2B	Adenovirus-transformed normal human bronchial epithelial cells	ATCC
NHBE	Normal human bronchial epithelial cells	Cambrex
BSMC	Normal human bronchial smooth muscle cells	Cambrex

* American Type Culture Collection