Enhanced formation of 5-oxo-6,8,11,14-eicosatetraenoic acid by cancer cells in response to oxidative stress, docosahexaenoic acid and neutrophil-derived 5-hydroxy-6,8,11,14-eicosatetraenoic acid

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The 5-lipoxygenase (5-LO) product 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), which is a potent chemoattractant for myeloid cells, is known to promote the survival of prostate cancer cells. In the present study, we found that PC3 prostate cancer cells and cell lines derived from breast (MCF7) and lung (A-427) cancers contain 5-hydroxyeicosanoid dehydrogenase (5-HEDH) activity and have the ability to synthesize 5-oxo-ETE from its precursor 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) when added as an exogenous substrate. H₂O₂ strongly stimulated the synthesis of 5-oxo-ETE and induced dramatic increases in the levels of both glutathione disulfide and NADP⁺. The effects of H₂O₂ on 5-oxo-ETE and NADP⁺ were blocked by *N*-ethylmaleimide (NEM), indicating that this effect was mediated by the glutathione reductase-dependent generation of NADP⁺, the cofactor required by 5-HEDH. 5-Oxo-ETE synthesis was also stimulated by agents that have cytotoxic effects on tumor cells, including 4,7,10,13,16,19-docosahexaenoic acid, tamoxifen and MK-886. Because PC3 cells have only modest 5-LO activity compared with inflammatory cells, we investigated their ability to contribute to the transcellular biosynthesis of 5-oxo-ETE from neutrophil-derived 5-HETE. Stimulation of neutrophils with arachidonic acid and calcium ionophore in the presence of PC3 cells led to a large and selective increase in 5-oxo-ETE synthesis compared with controls in which PC3 cell 5-oxo-ETE synthesis was selectively blocked by pretreatment with NEM. The ability of prostate tumor cells to synthesize 5-oxo-ETE may contribute to tumor cell proliferation as well as the influx of inflammatory cells, which may further induce cell proliferation through the release of cytokines. 5-Oxo-ETE may be an attractive target in cancer therapy.

the enzyme and is subsequently reduced by glutathione peroxidase to 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) (Figure 1). LTA₄ is converted to either LTB₄ or the cysteinyl LTs LTC₄ and LTD₄, whereas 5-HETE can be oxidized to 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) (2). The latter reaction is limited by the availability of the cofactor NADP⁺, which is normally present at only low concentrations in resting cells, which maintain a reducing environment with high levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (3). However, 5-oxo-ETE synthesis can be stimulated by oxidative stress (4) (Figure 1) and, in phagocytic cells, by the respiratory burst (5).

Because there was some evidence for a link between high fat diets and cancer (6), Ghosh *et al.* (7,8) examined the effects of AA and inhibitors of its metabolism on prostate tumor cell proliferation and survival. They found that AA stimulates PC3 and LNCaP prostate cancer cell proliferation. This effect was mimicked by 5-oxo-ETE and, to a lesser extent, by its precursor 5-HETE but not by other 5-LO products, including LTs B₄, C₄ and D₄ (8). Furthermore, both 5-LO inhibitors and FLAP antagonists, but not cyclo-oxygenase inhibitors, were found to induce apoptosis in these cells. The effects of the FLAP antagonist MK-886 were blocked by addition of 5-oxo-ETE but not by LTB₄ (7). 5-Oxo-ETE is known to act via the OXE receptor, which is a pertussis toxin-sensitive G-protein coupled receptor (9,10). 5-HETE has relatively weak biological activities that may be mediated either by a weak interaction with OXE receptor or by its metabolism to 5-oxo-ETE (11). This receptor has been identified in PC3 cells (12) as well as other tumor cell lines (13). Blocking expression of OXE receptor in PC3 cells with small interfering RNA led to reduced cell viability, suggesting that endogenously produced 5-oxo-ETE plays an important role in maintaining the survival of these cells (12).

Since 5-oxo-ETE could potentially be an important regulator of prostate cancer cell proliferation, we sought to determine whether these cells have the ability to synthesize this substance from its precursor 5-HETE and, if so, to investigate the regulation of this reaction. We have recently shown that dying neutrophils exhibit a dramatically increased ability to synthesize 5-oxo-ETE, and we hypothesized that cytotoxic agents could have similar effects on tumor cells. Finally, since abundant 5-LO activity is largely restricted to cells of the immune system, we wanted to determine whether PC3 cells could synthesize 5-oxo-ETE by transcellular biosynthesis from neutrophil-derived 5-HETE.

Introduction

Arachidonic acid (AA) is oxygenated by 5-lipoxygenase (5-LO) in the presence of the accessory protein, 5-lipoxygenase activating protein (FLAP), both of which are particularly abundant in inflammatory cells (1). The initial product of this reaction, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HpETE), is further converted by 5-LO to the unstable intermediate leukotriene (LT) A₄ or dissociates from

Abbreviations: AA, arachidonic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DHA, 4,7,10,13,16,19-docosahexaenoic acid; FLAP, 5-lipoxygenase activating protein; GSH, glutathione; GSSC, glutathione disulfide; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; 5-HETE, 5S-hydroxy-6,8,11,14-eicosatetraenoic acid; 13-HODE, 13S-hydroxy-9Z,11E-octadecadienoic acid; 5-HpETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LT, leukotriene; 5-LO, 5-lipoxygenase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NEM, *N*-ethylmaleimide; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase high-performance liquid chromatography.

Materials and methods

Materials

5-HETE (14) was prepared by total organic synthesis, whereas 13S-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) was produced by incubating linoleic acid (Nu-Chek Prep, Elysian, MN) with soybean lipoxygenase (Sigma-Aldrich, St Louis, MO) (15). Prostaglandin B₂ (PGB₂) was obtained from Cayman Chemical (Ann Arbor, MI), whereas AA and 4,7,10,13,16,19-docosahexaenoic acid (DHA) were from Nu-Chek Prep. All of the above products were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) before use.

Dimethyl sulfoxide, *o*-phthalaldehyde, tamoxifen, *N*-ethylmaleimide (NEM), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and Dextran 500 were obtained from Sigma-Aldrich. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Nepean, ON). Triacsin C and MK-886 were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) and Cayman Chemical, respectively. Ficoll-Paque was from Amersham Pharmacia Biotech (Montreal, Quebec, Canada), and NADP⁺ was from Roche Diagnostics (Laval, Quebec, Canada). Products used for cell culture, including cell growth media, antibiotics, fetal

Fig. 1. Metabolism of AA by the 5-LO pathway. AA is initially converted by 5-LO in the presence of FLAP to 5-HpETE, which is either further converted to LTA₄ by 5-LO or lost from the enzyme and converted to 5-HETE by glutathione peroxidase (GPx). LTA₄ is the precursor for LTs (LTB₄ and the cysteinyl LTs LTC₄ and LTD₄). 5-HETE is metabolized to 5-oxo-EETE by 5-HEDH in the presence of NADP⁺, the concentration of which is regulated by oxidative stress induced GSSG formation. The reduction of GSSG back to GSH by glutathione reductase (GRed) generates NADPH.

bovine serum and trypsin were purchased from Invitrogen (Burlington, Ontario, Canada).

Culture of tumor cell lines

The epithelial cancer cell lines, PC3, A-427 and MCF7 were obtained from the American Type Culture Collection and were cultured in RPMI [RPMI 1640 medium containing sodium bicarbonate (1.5 g/l), sodium pyruvate (1 mM) and L-glutamine (2 mM)], supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were grown in culture flasks until 80% confluent and then trypsinized (0.25% trypsin supplemented with ethylenediaminetetraacetic acid) and plated at a density of 1.5×10^4 cells/cm². Cells were subcultured to a maximum of 20 passages.

Preparation of neutrophils

Blood was obtained from healthy subjects with the approval of the Institutional Review Board of the McGill University Health Center. Neutrophils were prepared as described previously (2), using Dextran 500 to remove red blood cells followed by centrifugation over Ficoll-Paque to remove mononuclear cells and hypotonic lysis of any remaining red cells. The neutrophils were washed by centrifugation and resuspended in phosphate-buffered saline (PBS).

Analysis of eicosanoids by RP-HPLC

Eicosanoids were analyzed by precolumn extraction-RP-HPLC as described previously (16). Incubations with PC3, A427 and MCF7 cells were carried out in six-well plates (3×10^5 cells per well) at 37°C. Incubations were terminated by transferring the medium to a tube and washing the well with ice-cold methanol (0.63 ml), which was then added to the tube. The samples were then diluted with water to give a final concentration of 30% methanol and stored at -80°C prior to analysis.

HPLC analysis was performed using a modified Waters 2695 Alliance system (Waters Corporation, Mississauga, Ontario, Canada) with a photodiode array detector (Waters model 2996). A C18 SecurityGuard cartridge (4 × 3 mm; Phenomenex, Torrance, CA) was used for precolumn extraction. Either 13-HODE (100 ng; 5-HETE metabolites) or PGB₂ (100 ng; AA metabolites) was used as an internal standard. 5-HETE metabolites were separated on a 150 × 3.9 mm Nova-Pak C18 column (4 µm particle size; Waters, Milford, MA) maintained at 35°C, with a flow rate of 1 ml/min using a linear gradient between solvents A (0.02% acetic acid in water) and B (0.02% acetic acid in acetonitrile) as follows: 0 min: 65% B; 1.5 min: 65% B; 7 min: 82% B; 8 min: 82% B. AA metabolites were also analyzed on the above Nova-Pak column at 30°C using a gradient between solvents A, B and C (0.02% acetic acid in methanol) as follows: 0 min: 65% A, 24% B and 11% C; 4 min: 65% A, 24% B and 11% C; 39 min: 11% A, 38% B and 51% C.

Measurement of glutathione by RP-HPLC with postcolumn derivatization

Glutathione (GSH) and glutathione disulfide (GSSG) were measured by RP-HPLC as described previously (17). PC3 cells cultured on six-well plates (3×10^5 cells per well) were incubated in the presence or absence of H₂O₂ (100 µM) in PBS containing 1.8 mM Ca⁺⁺ and 1 mM Mg⁺⁺ (PBS⁺) at 37°C. Incubations were terminated after various times by rapid removal of the medium and addition 200 mM phosphoric acid (1 ml) containing 12 mM CHAPS at 0°C, followed by vigorous mixing with a pipette. GSH and GSSG were separated by RP-HPLC and detected by automated postcolumn derivatization using

o-phthalaldehyde at pH 12. The resulting isoindole adduct was detected using a fluorescence detector. The amounts of GSH and GSSG were determined from a standard curve with the authentic compounds as external standards.

Quantitation of NADP⁺ by RP-HPLC

PC3 cells (3×10^5 cells per well) in six-well plates were incubated in the presence or absence of H₂O₂ for various times in PBS⁺ at 37°C. The incubations were stopped by removal of the medium and addition of a mixture of acetophenone (50 mM) and KOH (3 N) in 50% MeOH (1 ml) at 0°C. Deamino-NAD⁺ (100 ng) was added as an internal standard. The samples were processed as described previously (17) and the resulting naphthyridine derivative was quantitated by RP-HPLC using a fluorescence detector.

Coincubation of PC3 cells with neutrophils

PC3 cells (6×10^5 cells per well) were preincubated for 10 min at 37°C with either vehicle or NEM (100 µM) in PBS⁺ (1 ml). The medium was then removed and the cells washed twice with PBS⁺, followed by the addition of either vehicle or H₂O₂ (final concentration 100 µM) in 0.9 ml PBS⁺. After 10 min, neutrophils (3×10^6 cells) were added along with AA (40 µM) and A23187 (5 µM) in 0.1 ml PBS⁺ and the samples were incubated at 37°C for 30 min. At this time, the medium containing neutrophils was removed and placed in a tube on ice. Ice-cold methanol (0.63 ml) was immediately added to the wells containing the adherent cells and then removed and added to the medium containing neutrophils. For comparison, neutrophils (3×10^6 cells) were preincubated with NEM (100 µM) in six-well plates for 10 min, followed by the addition of H₂O₂, AA and A23187. In this case, the NEM was not removed and H₂O₂ was added at the same time as AA and A23187. The incubations were terminated with methanol as described above.

Data analysis

The values shown are means ± standard errors of data from 'n' independent experiments, as indicated in the figure legends. The statistical significance of differences was determined by either one-way or two-way analysis of variance, using the Tukey test as a multiple comparison method.

Results

PC3 cells convert 5-HETE to 5-oxo-EETE and this is enhanced by oxidative stress

To determine whether PC3 cells possess 5-HEDH activity, these cells were incubated with 5-HETE for 40 min and the products analyzed by RP-HPLC. Under these conditions, only a small amount of 5-oxo-EETE (*t*_R 6.8 min) was detected (Figure 2A). However, addition of H₂O₂ (100 µM) resulted in a dramatic increase in its formation (Figure 2B). The time course for the formation of 5-oxo-EETE (Figure 2C) shows that H₂O₂ dramatically increased its levels at all time points. Preincubation of PC3 cells with NEM for 10 min reduced the amount of 5-oxo-EETE formed in the presence of H₂O₂ by >90% and also reduced baseline production from 5-HETE by ~50% (*P* < 0.05). The concentration-response relationship for H₂O₂ is shown in Figure 2D. 5-Oxo-EETE production was increased by ~3-fold by 10 µM H₂O₂ (*P* < 0.001),

Fig. 2. Formation of 5-oxo-EETE by PC3 cells in the presence and absence of H_2O_2 . PC3 cells (3×10^5 cells per well) were incubated with 5-HETE (2 μ M) and treated with either vehicle (A) or H_2O_2 100 μ M (B) for 40 min in the presence of PBS containing Ca^{++} and Mg^{++} . Products were analyzed by RP-HPLC as described in Materials and Methods. 13-HODE (100 ng) was used as an internal standard (Std). Abbreviation: 5o-EETE, 5-oxo-EETE. (C) Time course for 5-oxo-EETE formation following preincubation of PC3 cells with either vehicle (open circles, closed circles; $n = 5$) or 100 μ M NEM (inverted closed triangles, open triangles; $n = 3$) for 10 min and then incubation with either vehicle (open circles, inverted closed triangles) or H_2O_2 (closed circles, open triangles) for various times. (D) PC3 cells were incubated with 5-HETE (2 μ M) for 10 min in the presence of either vehicle or different concentrations of H_2O_2 ($n = 3$). The values are means $\pm$ standard errors.

which had an EC_{50} of ~ 40 μ M. We did not observe any other metabolites of 5-HETE or 5-oxo-EETE under these conditions, although it should be pointed out that we would not have been able to detect either 6,7-dihydro-5-oxo-EETE (18) or 5-oxo-7-glutathionyl-8,11,14-eicosatrienoic acid (19) as they do not absorb in the ultraviolet region of the spectrum.

Effects of H_2O_2 on the GSH redox cycle in PC3 cells

The inhibitory effect of NEM on 5-oxo-EETE formation suggested the involvement of the GSH redox cycle and $NADP^+$, in agreement with our previous data on endothelial cells (20) and airway epithelial cells (17). To further explore this relationship, we measured GSH, GSSG and $NADP^+$ in PC3 cells following addition of H_2O_2 . H_2O_2 elicited a dramatic increase in GSSG levels that was accompanied by a corresponding fall in GSH (Figure 3A). GSSG rose to 130 times resting levels by 30 s and to 200 times by 2 min. These effects were maximal between 2 and 7 min, after which time GSSG slowly dropped, but still remained markedly elevated after 20 min. Similarly, $NADP^+$ levels increased abruptly after addition of H_2O_2 and were >10 times greater than basal levels by 1 min (Figure 3B). Unlike GSSG, $NADP^+$ continued to increase and reached nearly 30 times control levels by 20 min. As with 5-oxo-EETE, the effects of H_2O_2 on $NADP^+$ were blocked by NEM.

Effects of cytotoxic agents on 5-oxo-EETE synthesis by tumor cells

We have recently shown that spontaneous cell death in neutrophils is associated with increased synthesis of 5-oxo-EETE (21). We wondered whether the initiation of cell death in tumor cells could also enhance the synthesis of 5-oxo-EETE, and therefore investigated the effects of a series of agents reported to have cytotoxic effects on these cells, including the $\omega 3$ polyunsaturated fatty acid DHA (22), the FLAP antagonist MK-886 (7) and tamoxifen (23). All three agents strongly stimulated 5-oxo-EETE synthesis from 5-HETE by PC3 cells, with DHA having the greatest effect (Figure 4A). 5-Oxo-EETE has been shown to be incorporated into cellular lipids (24) so one possible mechanism for the stimulatory effect of DHA on 5-oxo-EETE levels was that it competitively inhibited this process. However, triacsin C, an acyl CoA inhibitor that blocks 5-oxo-EETE uptake (24), had only a relatively small (although significant: $P < 0.05$) effect on 5-oxo-EETE levels (Figure 4A).

To determine whether similar responses are observed in other tumor cell lines, we examined 5-oxo-EETE synthesis by A-427 lung carcinoma cells (Figure 4B) and MCF7 breast adenocarcinoma cells (Figure 4C). 5-Oxo-EETE formation was strongly stimulated by DHA in both cell types and to a lesser extent by tamoxifen in A-427 cells.

Fig. 3. Effect of H_2O_2 on glutathione and $NADP^+$ levels in PC3 cells. The time courses for the effects of H_2O_2 (100 μ M) on (A) GSSG (closed circles) and GSH (open circles) levels ($n = 4$) and (B) $NADP^+$ levels in the presence of either vehicle (closed circles; $n = 6$) or 100 μ M NEM (open circles; $n = 2$) in PC3 cells (3×10^5 cells per well). GSH, GSSG and $NADP^+$ were measured by RP-HPLC. Values are means $\pm$ standard errors.

Transcellular biosynthesis of 5-oxo-EETE

To determine whether PC3 cells could synthesize 5-oxo-EETE from neutrophil-derived 5-HETE by transcellular biosynthesis, we stimulated mixtures of these cells with AA and A23187. One potential control for this experiment would have been to compare the amounts of 5-oxo-EETE synthesized by neutrophils alone in six-well plates to the amounts synthesized following addition of neutrophils to adherent PC3 cells. However, the ability of neutrophils to synthesize 5-LO products could easily be affected by the surfaces with which they interact (plastic versus PC3 cell surface). An ideal control would have been compare the effects of neutrophils added to PC3 cells which either did or did not contain 5-HEDH, but since the sequence of this enzyme is unknown, it was not possible to deplete it. Another possibility was to limit the ability of PC3 cells to make 5-oxo-EETE by blocking the GSH redox cycle. As shown in Figures 2C and 3B, NEM is a very efficient inhibitor of the generation of both $NADP^+$ and 5-oxo-EETE by PC3 cells in response to H_2O_2 .

To ensure that our experimental conditions would selectively affect 5-oxo-EETE synthesis in PC3 cells, we examined the effects of H_2O_2 and NEM on neutrophils incubated with AA and A23187 in the absence of PC3 cells (Figure 5). In agreement with our previous results (4), H_2O_2 had no effect on 5-oxo-EETE synthesis by neutrophils and this was not affected by NEM (Figure 5A). Neither did H_2O_2 affect the formation of 5-HETE and LTB_4 by neutrophils. In contrast, NEM significantly increased LTB_4 levels by ~ 2 -fold, in both the presence and absence of

Fig. 4. Effects of cytotoxic agents on 5-oxo-EETE formation by tumor cells. 5-HETE (4 μ M) was incubated with either PC3 (A), MCF7 (B) or A427 (C) cells (3×10^5 cells per well) in RPMI 1640 medium containing 0.5% fetal bovine serum in the presence of vehicle (open circles), 40 μ M DHA (closed circles), 10 μ M MK-886 (closed triangles), 50 μ M tamoxifen (open triangles; Tam) or 20 μ M Triacsin C (closed inverted triangles; TC). 5-Oxo-EETE was quantitated RP-HPLC. Values are means $\pm$ standard errors. 5-Oxo-EETE levels are given in nmol/ 10^6 cells, based on the numbers of cells present at the beginning of the incubation period. The numbers of independent determinations for PC3 cells were 9, 6, 4, 4 and 3 for vehicle, DHA, MK-886, tamoxifen and triacsin C, respectively. For A427 cells, $n = 7$ (vehicle and DHA) or 3 (tamoxifen) and for MCF7 cells, $n = 3$.

Fig. 5. Effects of NEM and H₂O₂ on the formation of 5-LO products by human neutrophils. Neutrophils (3×10^6 cells in 1 ml) were preincubated for 10 min with vehicle (solid bars) or NEM (hatched bars) followed by incubation with H₂O₂ (100 μ M), A23187 (5 μ M) and AA (40 μ M) for a further 30 min. The amounts of 5-oxo-EETE (A), 5-HETE (B) and LTB₄ (C) were determined by RP-HPLC ($n = 5$).

H₂O₂ ($P < 0.05$; Figure 5C) and tended to reduce 5-HETE synthesis, although this effect was not statistically significant (Figure 5B).

Because of the effects of NEM on neutrophils, in cocultivation experiments, PC3 cells were first incubated with NEM, which was then removed and the cells washed prior to preincubation with H₂O₂ and addition of AA, A23187 and neutrophils as shown in Figure 6A. In these experiments, the contribution of transcellular biosynthesis to the formation of 5-oxo-EETE was assessed by determining the amounts of 5-oxo-EETE that were formed in cocultures of neutrophils with GSH-replete PC3 cells compared with GSH-deficient (i.e. NEM pretreated) PC3 cells. H₂O₂ stimulated 5-oxo-EETE synthesis by cocultures of neutrophils with vehicle-treated PC3 cells (56% above control; $P < 0.05$; Figure 6B) but did not have a significant effect on the formation of either 5-HETE (Figure 6C) or LTB₄ (Figure 6D). Pretreatment of PC3 cells with NEM reduced 5-oxo-EETE synthesis by 75% in cocultures with neutrophils in the presence of H₂O₂ ($P < 0.001$) and by 55% in its absence ($P < 0.05$) (Figure 6B). In contrast, NEM had no effect on the synthesis of 5-HETE (Figure 6C) and LTB₄ (Figure 6D) by neutrophil/PC3 cell cocultures.

Discussion

The present study demonstrates that PC3 prostate cancer cells as well as cell lines derived from tumors in the lungs and breast possess high levels of 5-HEDH activity and can synthesize 5-

Fig. 6. Formation of 5-LO products in cocultures of PC3 cells with neutrophils. (A) PC3 cells (6×10^5 cells per well) were preincubated with vehicle or NEM followed by washing and incubation with either vehicle or H₂O₂ (100 μ M) for a further 10 min. Neutrophils (3×10^6 cells) along with A23187 (5 μ M) and AA (40 μ M) were then added and the cells incubated for an additional 30 min. The amounts of 5-oxo-EETE (B), 5-HETE (C) and LTB₄ (D) formed following preincubation of PC3 cells with either vehicle (solid bars) or NEM (100 μ M; hatched bars) were determined by RP-HPLC ($n = 5$).

oxo-EETE from its precursor 5-HETE. As we previously observed for human A549 cells, an alveolar epithelial-like cell line and normal human bronchial epithelial cells (17), 5-oxo-EETE synthesis is strongly stimulated by oxidative stress in the form of H₂O₂. The involvement of the GSH redox cycle in the response to H₂O₂ in PC3 cells was confirmed by the inhibitory effect of the sulfhydryl-alkylating agent NEM, which reduced the rate of 5-oxo-EETE synthesis to well below baseline values. This suggests that glutathione is an important regulator of 5-oxo-EETE synthesis in PC3 cells even under basal unstimulated conditions (Figure 1). The stimulatory effect of oxidative stress on 5-oxo-EETE formation correlated well with the dramatic and sustained increase in intracellular GSSG levels. This was coupled to a rapid increase in NADP⁺, which continued to rise for up to at least 20 min, presumably driven by the high levels of GSSG and its NADPH-dependent reduction to GSH by glutathione reductase (25). The involvement of the GSH

redox cycle in regulating NADP⁺ levels in PC3 cells was confirmed by the complete blockage of the increase in NADP⁺ by NEM.

When cultured in the presence of serum for 24 h, neutrophils undergo spontaneous apoptosis (26), which is associated with a large increase in their ability to synthesize 5-oxo-EETE (21). This effect appeared to be related to cell death and oxidative stress as it was inhibited by neutrophil survival factors such as granulocyte-macrophage colony-stimulating factor and antioxidants (21). Because 5-oxo-EETE production is regulated in PC3 cells by oxidative stress, which is associated with cell death, we sought to determine whether agents that have been reported to induce death of these cells could also stimulate 5-oxo-EETE synthesis. DHA has been reported to inhibit cell proliferation *in vitro* and to induce apoptosis in PC3 and other prostate tumor cell lines (22). Moreover, dietary DHA was found to be associated with a reduced incidence of prostate cancer (27). DHA may induce death in cancer cells by a number of mechanisms (28), including lipid peroxidation (29), perhaps mediated in part by down-regulation of superoxide dismutase (30). DHA stimulated 5-oxo-EETE synthesis in all three cell lines tested, suggesting that this is a fairly general response among tumor cells. This could be due to oxidative stress related to cell death and would be analogous to the increased ability of neutrophils undergoing spontaneous apoptosis to synthesize 5-oxo-EETE.

To determine whether other agents known to induce cancer cell death also increase 5-oxo-EETE synthesis, we examined the effects of MK-886 and tamoxifen. MK-886 is an FLAP antagonist that has been shown to induce apoptosis in tumor cells including PC3 cells. There is evidence that this may be due to inhibition of the formation of 5-oxo-EETE, which has been reported to be a survival factor for prostate tumor cells (7). However, the concentrations of MK-886 required for this effect are in the micromolar range, considerably higher than those required to block the formation of 5-LO products, and it is possible that it may act by additional mechanisms such as mitochondrial permeabilization (31). Tamoxifen is an estrogen receptor antagonist that is used to treat breast cancer but has also been found to induce apoptosis in PC3 cells by an estrogen-independent mechanism involving inhibition of protein kinase C and the induction of p21 (23). Both agents stimulated 5-oxo-EETE synthesis in PC3 cells, possibly due to oxidative stress associated with cell death as noted above.

Although 5-oxo-EETE has previously been reported to promote cancer cell proliferation and to inhibit apoptosis (7,13), in the present study, nearly all of the cells had detached 24 h after addition of DHA and the other cytotoxic agents, despite the presence of 5-HETE. Moreover, the cells appeared to be quite fragile by 6 h as few intact cells could be recovered from the wells after scraping with a rubber policeman. Although our study was not designed to determine whether the cells died as a result of apoptosis or necrosis, all three of the cytotoxic agents used have all been reported to induce apoptosis of PC3 cells as noted above. Under the conditions employed, the concentration of 5-oxo-EETE in the wells rose to maximal levels in the range of 500 nM by 6 h (Figure 4A). There are several possible explanations for the failure of 5-HETE/5-oxo-EETE to block cell death. In the case of DHA, it has been reported that this fatty acid acts as an antagonist at the OXE receptor (10) and could thereby block any antiapoptotic effects of 5-oxo-EETE. It is also possible that the concentrations of 5-oxo-EETE reached during the initial 1 or 2 h of the incubations were too low to prevent the initiation of the apoptotic pathway. Alternatively, the antiapoptotic effects of 5-oxo-EETE were previously studied over a shorter 4 h time period (7), whereas our studies were >24 h. 5-Oxo-EETE could have reduced the rate of progression of cells into apoptosis at earlier time points but may have been unable to prevent their eventual death. In the case of MK-886, it would seem certain that any endogenous synthesis of this substance would have been blocked. However, at the relatively high concentration used in this and other studies, as noted above, it may have acted by additional mechanisms that could not be reversed by 5-oxo-EETE, at least over the 24 h period investigated in the present study. Nevertheless, the increased ability of dying cancer cells to synthesize 5-oxo-EETE

could potentially reduce the efficacy of cytotoxic agents, which, unlike MK886, do not already block its synthesis. In this situation, it is possible that concomitant administration of a 5-oxo-EETE receptor antagonist or synthesis inhibitor could have beneficial effects.

We did not detect 5-LO products after incubation of PC3 cells alone, in the absence of inflammatory cells, with AA and the calcium ionophore A23187, followed by HPLC analysis (data not shown). However, this method would not be sufficiently sensitive to detect low levels of these products. PC3 cells have been reported to release small amounts of the precursor for 5-oxo-EETE, 5-HETE (~1 ng/10⁶ cells), detected by immunoassay following culture for 24 h (7). Similar amounts of 5-HETE were detected by liquid chromatography-mass spectrometry after incubation of PC3 cells with AA in the presence of calcium ionophore for 10 min (32). However, there are no previous reports of 5-oxo-EETE synthesis by these cells. Further support for the existence of the 5-LO pathway in PC3 cells is the presence of 5-LO protein (32) as well as messenger RNA for both 5-LO and FLAP (33).

Because of the high 5-HEDH activity in PC3 cells and their very limited 5-LO activity, we sought to determine whether these cells could contribute to 5-oxo-EETE formation by transcellular biosynthesis using leukocyte-derived 5-HETE. The ideal control for such experiments would be PC3 cells that did not contain 5-HEDH, but this was not possible, as this enzyme has not yet been cloned and its sequence is unknown. Instead, we took advantage of the requirement of 5-HEDH for glutathione reductase-derived NADP⁺ (Figure 1) and inhibited this pathway by alkylating intracellular GSH with NEM, which could readily be removed prior to the addition of neutrophils. As shown in Figure 2C, NEM inhibits both basal and H₂O₂-stimulated 5-oxo-EETE synthesis from 5-HETE by PC3 cells but has no effect on 5-oxo-EETE formation by neutrophils (Figure 5A). The difference in 5-oxo-EETE formation when neutrophils were incubated with NEM-treated versus vehicle-treated PC3 cells represents the minimum contribution of PC3 cells to 5-oxo-EETE synthesis in these incubations. However, this is probably an underestimate because NEM does not completely inhibit the synthesis of 5-oxo-EETE by PC3 cells (Figure 2C). Thus, in the absence of H₂O₂, PC3 cells account for at least 50% of the 5-oxo-EETE produced in neutrophil-PC3 cell incubations, whereas in the presence of H₂O₂, they account for at least 75% (Figure 6A). This is the first demonstration that 5-oxo-EETE can be synthesized by transcellular biosynthesis. It highlights the potential importance of 5-HEDH activity in structural cells, including vascular endothelial cells (20) and airway epithelial and smooth muscle cells (17), that contain little or no 5-LO activity. These cells could also contribute to 5-oxo-EETE formation when exposed to 5-HETE released from activated leukocytes cells at sites of inflammation.

Although neutrophils were not exposed to NEM during the co-incubation experiments, we tested its effects on these cells for comparison. In contrast to PC3 cells, NEM had no effect on the synthesis of 5-oxo-EETE by neutrophils but increased LTB₄ formation and tended to reduce 5-HETE formation. The lack of effect of NEM on 5-oxo-EETE synthesis is not surprising, as we previously found that the GSH redox pathway does not play a major role in regulating this process in neutrophils (4), in contrast to NADPH oxidase, which has a strong stimulatory effect (5). The apparent inverse relationship between 5-HETE and LTB₄ in neutrophils is intriguing and could possibly be explained by reduced GSH peroxidase activity due to the depletion of GSH by NEM. This could result in a shift in the metabolism of 5-HpETE, favoring conversion to LTA₄ over peroxidase-catalyzed conversion to 5-HETE. Another explanation for the increased amounts of LTB₄ would be inhibition of LTB₄ 20-hydroxylase. The LTB₄ metabolite formed by this enzyme (20-hydroxy-LTB₄) was observed following incubation of vehicle-pretreated but not NEM-pretreated neutrophils with AA and A23187.

Transcellular biosynthesis appears to be an important mechanism for the formation of a variety of eicosanoids (34) in addition to 5-oxo-EETE. For example, endothelial cells can use platelet-derived

prostaglandin H₂ in addition to endogenously derived prostaglandin H₂ to synthesize prostaglandin I₂ (35). A variety of cell types with little or no 5-LO activity have been shown to synthesize LTs from neutrophil-derived LTA₄. Red blood cells, which contain LTA₄ hydro-lase but not 5-LO, can synthesize LTB₄ in the presence of activated neutrophils (36), whereas platelets, which contain LTC₄ synthase but not 5-LO, can synthesize LTC₄ from neutrophil-derived LTA₄ (37). An elegant study by Fabre *et al.* (38) demonstrated the importance of transcellular biosynthesis for the synthesis of LTB₄ *in vivo* in animal models of inflammation. A number of instances of transcellular bio-synthesis involving the release of AA from one cell type and its conversion to cyclo-oxygenase and 5-LO products by a different cell type have also been described (34).

Substantial numbers of inflammatory cells are present in tumors, including both neutrophils (39) and eosinophils (40). Although in some cases these cells may have antitumor effects, in many cases, they promote tumor progression due to the release of chemoattractants, cytokines and reactive oxygen species, which can promote cell proliferation, further infiltration of inflammatory cells and angiogenesis (41,42). Macrophages, neutrophils and eosinophils all have high levels of 5-LO and, in addition to synthesizing 5-oxo-EETE directly, could provide 5-HETE to 5-HEDH in tumor cells, resulting in the formation of 5-oxo-EETE by transcellular biosynthesis. This could be enhanced in necrotic areas of the tumor as the present study demonstrates that tumor cell death is associated with an increased ability to synthesize 5-oxo-EETE. Reactive oxygen species produced by infiltrating inflammatory cells would also promote 5-oxo-EETE synthesis.

Because of its proliferative effect on tumor cells (7), 5-oxo-EETE produced within necrotic areas of tumors could contribute to cell growth in adjacent areas. It could also serve as a chemoattractant to induce the infiltration of eosinophils (43), neutrophils (44), monocytes (45) and possibly basophils (46). Eosinophils express high levels of the OXE receptor for 5-oxo-EETE (9) and are very prominent within some tumors (40). There is an evidence that dying tumor cells release an eosinophil chemoattractant (40), and 5-oxo-EETE would be an excellent candidate for such a substance. The increased production of 5-oxo-EETE in response to cytotoxic agents could reduce their effectiveness, especially in the presence of inflammatory cells. This raises the possibility that drugs targeting 5-oxo-EETE by preventing its synthesis or actions could be beneficial adjuncts in the treatment of cancer, especially considering the antiproliferative effect of small interfering RNA directed at the OXE receptor (12).

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