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Oxidative stress-induced changes in pyridine nucleotides and chemoattractant 5-lipoxygenase products in aging neutrophils

François D. Graham^{*}, Karl-Rudolf Erlemann^{*,1}, Sylvie Gravel^{*}, Joshua Rokach[†], and William S. Powell^{*}

^{*}Meakins-Christie Laboratories, McGill University, Montreal, QC, Canada H2X 2P2

[†]Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, FL 32901-6982, USA

Abstract

Neutrophils spontaneously undergo apoptosis, which is associated with increased oxidative stress. We found that there is a dramatic shift in the formation of 5-lipoxygenase products during this process. Freshly isolated neutrophils rapidly convert leukotriene B₄ (LTB₄) and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) to their biologically inactive ω -oxidation products. However, ω -oxidation is impaired in neutrophils cultured for 24 h, when only 25% of the cells are nonapoptotic, resulting in the persistence of LTB₄ and a dramatic shift in 5-HETE metabolism to the potent granulocyte chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-EETE). The reduced ω -oxidation activity appears to be due to a reduction in LTB₄ 20-hydroxylase activity, whereas the increased 5-oxo-EETE formation was caused by a dramatic increase in the 5-hydroxyeicosanoid dehydrogenase cofactor NADP⁺. NAD⁺, but not NADPH, also increased, as did the GSSG/GSH ratio, indicative of oxidative stress. The changes in 5-HETE metabolism and pyridine nucleotides were inhibited by anti-apoptotic agents (GM-CSF, forskolin) and antioxidants (diphenylene iodonium, catalase, deferoxamine), suggesting the involvement of H₂O₂ and possibly other reactive oxygen species. These results suggest that in severe inflammation, aging neutrophils that have evaded rapid uptake by macrophages may produce increased amounts of the chemoattractants 5-oxo-EETE and LTB₄, resulting in delayed resolution or exacerbation of the inflammatory process.

Keywords

Eicosanoids; Arachidonic acid; 5-Oxo-EETE; 5-HETE; Leukotriene B₄; Neutrophils; Oxidative stress; NADP⁺; NADPH; Glutathione; Cell death; Survival factors; Antioxidants; High performance liquid chromatography

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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 William.Powell@McGill.ca.

¹Present address: Karl-Rudolf Erlemann, InSymbiosis Discovery Inc, 4141 Sherbrooke Street West, Suite 410, Montreal, QC, H3Z 1B8, Canada

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Introduction

Neutrophils have been implicated in many inflammatory diseases [1,2] and can elicit tissue injury by the release of reactive oxygen species (ROS)², lysosomal enzymes and proinflammatory cytokines. These cells possess high levels of 5-lipoxygenase (5-LO), which converts arachidonic acid (AA) to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HpETE) and leukotriene (LT) A₄ (Fig. 1) [3]. LTA₄ is metabolized to LTB₄ by LTA₄ hydrolase, whereas 5-HpETE is reduced to 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by peroxidase. LTB₄ is a potent chemoattractant for neutrophils and other leukocytes [4], but is rapidly metabolized to its ω -oxidation product 20-hydroxy-LTB₄ by LTB₄ 20-hydroxylase (CYP4F3A) in the presence of NADPH [5], resulting in a nearly 10-fold reduction in biological potency [6]. 5-HETE, on the other hand, has only weak biological activity on neutrophils, but is oxidized by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) to 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-EETE), a potent activator of human neutrophils and eosinophils [7]. Both 5-HETE [8] and 5-oxo-EETE [9] are converted to biologically inactive 20-hydroxy metabolites by LTB₄ 20-hydroxylase.

5-HEDH is a microsomal enzyme that is highly selective for 5-HETE and requires NADP⁺ as a cofactor [10]. It is found in most types of inflammatory cells [7] as well as airway epithelial and smooth muscle cells [11] and vascular endothelial cells [12]. The synthesis of 5-oxo-EETE is dependent not only on 5-HEDH, but also on NADP⁺ levels, which are normally very low in unstimulated cells, in contrast to the high levels of its reduced counterpart, NADPH [13]. Thus unstimulated neutrophils convert 5-HETE to only small amounts of 5-oxo-EETE, even though they possess high levels of 5-HEDH, but instead form 5,20-diHETE [8,9] (Fig. 1). However, 5-oxo-EETE synthesis is rapidly increased in neutrophils and other phagocytes by activation of NADPH oxidase (NOX2) [9], and in a variety of cells by oxidative stress [7].

The actions of LTB₄ and 5-oxo-EETE are mediated by the BLT₁ [14] and OXE [15,16] G protein-coupled receptors, which are highly selective for their respective ligands. A variety of responses are induced in granulocytes by 5-oxo-EETE, including calcium mobilization, actin polymerization, adhesion molecule expression, and cell migration [7]. This substance is also a potent stimulator of degranulation and superoxide production in granulocytes that have been primed with GM-CSF or TNF α [17]. 5-Oxo-EETE elicits transendothelial migration of eosinophils [18] and, when administered *in vivo*, induces pulmonary eosinophilia in rats and infiltration of eosinophils and neutrophils into the skin in humans [7].

Neutrophils are short-lived and undergo spontaneous apoptosis, which may be associated with oxidative stress [19]. Oxidative stress augments 5-LO activity in certain cells, such as B lymphocytes [20] and increases 5-oxo-EETE formation from 5-HETE [11]. We therefore hypothesized that cultured neutrophils undergoing apoptosis might synthesize greater amounts of the potent proinflammatory mediators 5-oxo-EETE and LTB₄. To test this hypothesis we investigated the effects of aging neutrophils in culture on the metabolism of AA and 5-HETE as well as pyridine nucleotide levels. We found that after 24 h in culture, neutrophils have elevated levels of NADP⁺ and produce much greater amounts of 5-oxo-EETE and LTB₄. These changes appear to be mediated by oxidative stress associated with apoptosis.

²The abbreviations used are: 13-HODE, 13S-hydroxy-9Z,11E-octadecadienoic acid; 15-HETE, 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 5,20-diHETE, 5,20-dihydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; 5-HETE, 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-HpETE, 5-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; 5-oxo-20-HETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-oxo-EETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; AA, Arachidonic acid; DPI, diphenylene iodonium; GM-CSF, granulocyte macrophage-colony stimulating factor; LT, leukotriene; NOX2, NADPH oxidase-2; ODS, octadecylsilyl; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide; PMS phenazine methosulfate; ROS, reactive oxygen species; RP-HPLC, reversed-phase-high performance liquid chromatography; SOD, superoxide dismutase; TBAH, tetrabutyl ammonium hydroxide.

Materials and Methods

Materials

5-Oxo-ETE [21] and LTB₄ [22] were prepared by total chemical synthesis. 13S-hydroxy-9Z, 11E-octadecadienoic acid (13-HODE) was prepared by oxidation of linoleic acid with soybean lipoxygenase Type 1B (Sigma-Aldrich, St. Louis, MO) as described in the literature [23]. AA was from Nu-Chek Prep, Inc, Elysian, MN and was purified by reversed-phase high performance liquid chromatography (RP-HPLC) before use.

Lucigenin, bovine liver catalase, deferoxamine, Dextran 500 (from Leuconostoc), DMSO, diphenyliodonium chloride (DPI), EDTA, forskolin, glutathione, NAD⁺, deamino-NAD⁺, phorbol-12 myristate-13-acetate (PMA), phenazine methosulfate (PMS), bovine erythrocyte superoxide dismutase (SOD), and tetrabutyl ammonium hydroxide (TBAH) were purchased from Sigma-Aldrich. RPMI 1640 and other cell culture materials were obtained from Invitrogen (Burlington, ON). NADP⁺, NADH, and NADPH were from Roche Diagnostics (Indianapolis, IN). Dithiothreitol and phenylmethylsulfonyl fluoride were purchased from Boehringer-Mannheim (Mannheim, Germany) and ICN Biomedicals Inc. (Aurora, OH), respectively, whereas recombinant human GM-CSF was from Peprotech, Rocky Hill, NJ. Ficoll-Paque was obtained from GE Healthcare Bio-Sciences, Uppsala, Sweden. HPLC solvents were from Fisher Scientific, Nepean, ON. FK866 was kindly provided by the NIMH.

Preparation and culture of neutrophils

Neutrophils were purified from whole blood from healthy human subjects as described previously 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 [10]. The neutrophils were washed by centrifugation in PBS and either suspended in PBS or cultured for 24 h in RPMI 1640 containing 10% FBS, sodium bicarbonate (1.5 g/l), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). After this time the cells were centrifuged and suspended in PBS.

Preparation of neutrophil microsomes

Neutrophils (4×10^8 cells) were resuspended in PBS (20 ml) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 0.5 mM dithiothreitol, and three protease inhibitor cocktail tablets (Roche Diagnostics, Laval QC). Cells were sonicated in an ice bath (Sonics and Materials, model 4710) with a setting of 40 cycles/s (4×6 s pulsing, with 1 min between cycles). Neutrophil sonicates were centrifuged at $1500 \times g$ for 10 min at 4 °C and the supernatant centrifuged at $10,000 \times g$ for 10 min. The resulting supernatant was centrifuged at $153,000 \times g$ for 2 h and the pellet resuspended in PBS. Microsomal protein levels were determined using a modified Lowry assay.

Analysis of eicosanoids

Eicosanoids were quantitated by automated precolumn extraction/RP-HPLC as described previously [24]. Neutrophils (2×10^6 cells) or neutrophil microsomes (30 µg) were incubated at 37 °C with substrates (AA, 5-HETE, or LTB₄) in 1 ml PBS containing 1.8 mM Ca⁺⁺ and 1 mM Mg⁺⁺. Incubations were terminated with methanol containing 0.15% trifluoroacetic acid and cooling to 0 °C. Prior to analysis the pH was adjusted to 7 with 1 M NaH₂PO₄. 13-HODE (100 ng) or PGB₂ (100 ng) were used as internal standards. HPLC was performed using a Waters Alliance system (Waters Associates, Milford, MA). The stationary phase was a Novapak C18 column (3.9×150 mm; Waters). A C18 SecurityGuard cartridge (4×3 mm; Phenomenex, Torrance, CA) was used for precolumn extraction. The mobile phases are described in the figure legends.

Quantitation of NADP⁺ and NAD⁺

NADP⁺ and NAD⁺ were measured after conversion to fluorescent naphthyrindine derivatives followed by quantitation by RP-HPLC [25]. To a suspension of neutrophils (2×10^6 cells in 250 μ l PBS) was added a mixture of acetophenone (50 mM) and KOH (3 N) along with deamino-NAD⁺ (30 ng; internal standard) in 250 μ l of MeOH/H₂O (1:1). After 20 min at 0 °C, formic acid (62.5 μ l) was added and the samples stored at -80 °C until analysis (<2 wk). After thawing, the samples were extracted with ethyl acetate and the aqueous phases treated with 100 μ M PMS. Aliquots (50 μ l) were analyzed by RP-HPLC on an Ultracarb ODS column (Phenomenex) using a Waters model 2475 Multiwavelength Fluorescence Detector. The mobile phase was a gradient between (a) 100 mM citric acid containing 4 mM TBAH and (b) acetonitrile as follows: 0 min, 1% acetonitrile; 12 min, 25% acetonitrile; 15 min, 25% acetonitrile). The flow rate was 1.25 ml/min.

Measurement of NADPH and NADH

NADPH and NADH were measured by RP-HPLC, using a fluorescence detector [25]. Na₃PO₄ (200 mM; 50 μ l) was added to neutrophils (7.5×10^5 cells in 100 μ l PBS) and an aliquot (50 μ l) was analyzed by RP-HPLC using a Gemini column (3 μ m particle size; 150 $\times$ 3 mm; Phenomenex). The mobile phase was a gradient between solvent A (200 mM ammonium acetate containing 10 mM TBAH in water, adjusted to pH 6.0 with acetic acid and solvent B (200 mM ammonium acetate containing 10 mM TBAH in 30% methanol) as follows: 0 min, 20% B; 15 min, 100% B. The flow rate was 0.5 ml/min. NADPH was detected using a fluorescence detector (λ_{ex} , 325 nm; λ_{em} , 450 nm). The amounts of NADPH and NADH were calculated from a standard curve obtained using external standards.

Analysis of GSH and GSSG

GSH and GSSG were measured by RP-HPLC using post-column derivatization and a fluorescence detector [12]. Phosphoric acid (150 μ l; 200 mM) containing 12 mM CHAPS was added to a suspension of neutrophils (3×10^5 cells in 150 μ l). Aliquots (50 μ l) were analyzed by RP-HPLC using the above Ultracarb column. The mobile phase was a gradient between 0 and 15% acetonitrile in water containing 0.05% trifluoroacetic acid with a flow rate of 1 ml/min. GSH and GSSG in the column eluate were converted to a fluorescent isoindole adduct by introduction of a mixture of o-phthalaldehyde and Na₃PO₄, pH 12. The amounts of GSH and GSSG were determined using the authentic compounds as external standards.

Evaluation of superoxide production

To ensure that SOD and DPI were still effective after 24 h, freshly isolated neutrophils were cultured in 6 well plates (10^6 cells/well) in RPMI containing 10% FBS and either SOD, DPI or vehicle. After 24 h, the cells were incubated with 250 μ M lucigenin and stimulated with PMA (50 nM). Luminescence was measured in a luminometer (LUMAT LB 9507) every 30 s for 10 min. Superoxide production was assessed by calculating the area under the luminescence curve.

Evaluation of neutrophil survival

Neutrophil survival was estimated using an “annexin V FITC apoptosis detection kit” from Beckman/Coulter. Neutrophils (10^5 cells) were washed by centrifugation ($500 \times g/5$ min) and processed according to the manufacturer's directions. The samples were analyzed by flow cytometry for annexin V-FITC and propidium iodide (PI) binding within 30 min.

Data analysis

All values are means $\pm$ SE of data from “n” independent experiments, as indicated in the figure legends. The statistical significance of differences was determined by one-way or two-way ANOVA as appropriate, using the Bonferroni test as a multiple comparison method. Data from experiments comparing the effects of different treatments on neutrophils cultured for 24 h are expressed as percentages of controls, because it was not possible to include all of the treatments in each experiment.

Results

Formation of 5-LO products by cultured neutrophils

Neutrophils were incubated with the calcium ionophore A23187 for up to 24 h in the presence of 0.5% FBS and the major products were quantitated by RP-HPLC. After 20 min the major products were 5-HETE > LTB₄ > 5-oxo-EETE (Fig. 2). However, the concentrations of 5-HETE and LTB₄ declined at longer time points, whereas that of 5-oxo-EETE rose, so that by 24 h it was the major product. These results suggested that the production of 5-oxo-EETE by neutrophils was sustained over a long period of time in culture, when the cells would be expected to be undergoing apoptosis.

The preliminary experiment shown in Fig. 2 raised the possibility that neutrophils cultured for up to 24 h may have the ability to synthesize substantial amounts of 5-oxo-EETE. To investigate this more precisely, freshly isolated neutrophils and neutrophils that had been cultured for 24 h in the presence of 10% FBS were incubated with A23187 and AA for 2 h and the products quantitated. The main products formed by freshly isolated neutrophils were 20-hydroxy-LTB₄, 5-HETE, 5-oxo-EETE, and 15-HETE (Fig. 3A). Only a very small amount of LTB₄ was detected at this time point. Small amounts of 5-oxo-20-HETE (oh) and 5,20-diHETE(dh) were also detected, but the latter compound was not well separated from the internal standard PGB₂. In contrast, neutrophils that had been cultured for 24 h gave rise to much higher levels of 5-oxo-EETE and LTB₄ and somewhat diminished levels of 5-HETE and ω -oxidation products (Fig. 3B).

The time courses for the formation of 5-LO products are shown in Fig. 3C and 3D. 5-HETE was formed very rapidly by both freshly isolated and cultured neutrophils, reaching maximal levels by 5 min, followed by a sharp decline, whereas 5-oxo-EETE was formed more slowly, with maximal levels being reached by ~30 to 60 min. The levels of 5-HETE were substantially lower at all times in cultured neutrophils ($p < 0.001$), whereas those for 5-oxo-EETE were about twice those in freshly isolated neutrophils ($p < 0.001$). Like 5-HETE, LTB₄ was produced rapidly by freshly isolated neutrophils, reaching a maximum by 5 min and then declining (Fig. 3C). In cultured neutrophils the initial levels of LTB₄ were significantly lower after 5 min ($P < 0.05$), but its rate of decline was much slower, so that by 60 min, its levels were about three times higher than with freshly isolated cells ($p < 0.02$). This may have been due to reduced metabolism of LTB₄ after 24 h in culture, as the levels of 20-hydroxy-LTB₄ were slightly lower ($p < 0.001$).

Altered metabolism of 5-HETE by aging neutrophils

To further investigate the apparent changes in 5-HEDH and ω -oxidation activities in aging neutrophils, these cells were incubated with 5-HETE (2 μ M) for 30 min and the products quantitated. Freshly isolated neutrophils converted 5-HETE principally to the ω -oxidation product 5,20-diHETE, along with only small amounts of 5-oxo-EETE (Fig. 4A). In contrast, the most abundant 5-HETE metabolite formed by neutrophils cultured for 24 h was 5-oxo-EETE, along with smaller amounts of 5,20-diHETE and 5-oxo-20-HETE (Fig. 4B). Overall, cultured

neutrophils produced about 17 times more 5-oxo-EETE and only about one-fifth as much 5,20-diHETE ($p < 0.001$) after 30 min compared with freshly isolated cells (Fig. 4C).

The changes in 5-oxo-EETE formation could be due either to increased 5-HEDH activity or increased NADP⁺ levels. To estimate total 5-HEDH activity, neutrophils were preincubated with PMS to convert intracellular NADPH to NADP⁺ [9,26] prior to addition of 5-HETE. Under these conditions, the rates of 5-oxo-EETE synthesis were nearly identical in freshly isolated and cultured cells (Fig. 5A). Furthermore, microsomal 5-HEDH activity in the presence of excess NADP⁺ was the same in microsomes from freshly isolated and cultured neutrophils (Fig. 5B). The high rate of 5-oxo-EETE synthesis in cultured neutrophils (~50% of the maximal rate in PMS-treated cells) suggested that NADP⁺ may have risen dramatically during culture.

The reduced formation of ω -oxidation products of LTB₄ and 5-HETE by neutrophils after 24 h in culture could have been due to a decrease in either LTB₄ 20-hydroxylase or its cofactor NADPH. In contrast to 5-HEDH, microsomal LTB₄ 20-hydroxylase activity in the presence of excess NADPH was reduced by nearly 60% ($p < 0.02$) following culture of neutrophils for 24 h (Fig. 5C).

Pyridine nucleotide levels change dramatically in cultured neutrophils

To determine whether the changes in 5-oxo-EETE synthesis could be explained by altered pyridine nucleotide levels we measured these substances by RP-HPLC. NADP⁺ levels were very low in freshly isolated neutrophils (Fig. 6A), but increased dramatically after 24 h in culture (Fig. 6B). Overall, NADP⁺ levels rose almost 20-fold after 24 h ($p < 0.001$; Fig. 6C). Surprisingly, this was not mirrored by a reduction in NADPH, which tended to increase slightly after 24 h (not significant). Interestingly, the elevated NADP⁺ levels were accompanied by a parallel nearly 10-fold increase in NAD⁺ (Fig. 6D). NADH levels were also markedly higher after 24 h.

Impact of NAD⁺ on 5-oxo-EETE synthesis

The dramatic increase in 5-oxo-EETE synthesis that we observed after 24 h could potentially be due to the large increase in the levels of NAD⁺ and its conversion to NADP⁺ by NAD kinase. To evaluate this possibility, neutrophils were cultured for 24 h in the presence of either vehicle or FK866, which depletes cellular NAD⁺ [27]. This treatment lowered NAD⁺ and NADH levels to ~10% of those in vehicle-treated cells (Fig. 7A), whereas NADP⁺ and NADPH were reduced to ~25% of control levels. Despite the lower NAD⁺ and NADP⁺ levels, FK866 had no effect on the synthesis of 5-oxo-EETE or 5,20-diHETE (Fig. 7B). Nor did it affect total 5-HEDH activity in the presence of PMS.

Cell survival and redox status of cultured neutrophils

Because the changes in 5-oxo-EETE synthesis and pyridine nucleotide levels in cultured neutrophils could have been due to cell death and/or oxidative stress, we examined cell survival and redox status in cells after 24 h in culture. Measurement of annexin V and PI staining by flow cytometry in neutrophils revealed that after 24 h in culture, only 27% of the cells were unstained (Fig. 8B) compared to 96% of freshly isolated cells (Fig. 8A). After 24 h, the majority of cells (60%) were annexin V positive/PI negative (apoptotic cells) whereas a smaller number (13%) were double positive (late apoptotic/necrotic). Overall, only ~25% of the neutrophils were nonapoptotic after 24 h compared to about 95% of freshly isolated cells (Fig. 8C).

To evaluate the redox status of neutrophils glutathione levels were measured by RP-HPLC. Compared to freshly isolated neutrophils, GSH levels were ~50% lower ($p < 0.001$; Fig. 8D),

GSSG was higher ($p < 0.02$; Fig. 8E) and the percent of total glutathione that was oxidized was ~3.3-fold higher ($p < 0.001$) after 24 h (Fig. 8F).

Anti-apoptotic agents inhibit changes in 5-oxo-ETE and NADP⁺ in aging neutrophils

GM-CSF and forskolin, which promote neutrophil survival by different mechanisms [28,29], nearly doubled neutrophil survival after 24 h, from about 25% in vehicle-treated cells to nearly 50% ($p < 0.005$; Fig. 9A). They also inhibited 5-oxo-ETE synthesis by over 60% ($p < 0.01$; Fig. 9B) without affecting total 5-HEDH activity in the presence of PMS (Fig. 9D). GM-CSF, but not forskolin, increased 5,20-diHETE formation by about 2-fold ($p < 0.001$) (Fig. 9C). Both GM-CSF and forskolin lowered the ratio of NADP⁺/NADPH by over 60% ($p < 0.005$; Fig. 9E). With GM-CSF, this was due to a combination of lower NADP⁺ levels (~50%; $p < 0.001$) and higher NADPH levels (~200%; $p = 0.001$), whereas with forskolin, the only significant change was a nearly 70% reduction in NADP⁺ ($p < 0.02$) (data not shown). GM-CSF also increased NAD⁺ in neutrophils (Fig. 9F) and reduced GSSG levels (Fig. 9H).

Effects of antioxidants on pyridine nucleotides and 5-oxo-ETE synthesis

The increased glutathione oxidation after 24 h and its reversal by GM-CSF suggested a possible relationship between ROS production and 5-oxo-ETE synthesis. This was further investigated by examining the effects of various antioxidants: DPI, which inhibits NOX2, SOD, which degrades superoxide, catalase, which reduces H₂O₂ to H₂O, and deferoxamine, an iron chelator.

DPI strongly inhibited 5-oxo-ETE formation (Fig. 9B) without affecting total 5-HEDH activity (Fig. 9D). It also completely blocked the ω -oxidation of 5-HETE (Fig. 9C), presumably due to its potent inhibitory effect on LTB₄ 20-hydroxylase [9]. DPI reduced NADP⁺ levels by ~45% ($p < 0.001$) without affecting NADPH levels, resulting in a decline in the ratio of NADP⁺/NADPH ($p < 0.02$; Fig. 9E). It reduced GSH (Fig. 9G) but did not have a significant effect on either GSSG (Fig. 9H) or neutrophil survival (Fig. 9A).

SOD did not affect any of the parameters measured, except for a slight increase in NAD⁺. To ensure that the SOD was still active after 24 h, we tested its ability to block PMA-induced superoxide formation. This response was inhibited by $90 \pm 3\%$ in neutrophils cultured for 24 h in the presence of SOD compared to vehicle-treated cells (data not shown). DPI was also effective after 24 h, inhibiting PMA-induced superoxide formation by $97 \pm 2\%$.

Catalase and deferoxamine had effects nearly identical to GM-CSF. Neutrophils cultured for 24 h in the presence of these agents exhibited markedly increased survival, reduced 5-oxo-ETE synthesis and increased synthesis of 5,20-diHETE compared to vehicle-treated cells (Fig. 9). The ratio of NADP⁺ to NADPH was sharply reduced by both antioxidants. GSH levels were slightly higher and GSSG levels were lower in both cases, but these differences were statistically significant only for catalase. However, both antioxidants significantly reduced the ratio of GSSG to GSH ($p < 0.01$; data not shown).

Relationship between 5-oxo-ETE synthesis, pyridine nucleotides, and oxidized glutathione

To examine the correlation between the synthesis of 5-oxo-ETE and the ratio of NADP⁺ to NADPH, the individual data points for all of the experiments shown in Fig. 9 were plotted. As shown in Fig. 10A, 5-oxo-ETE levels were positively correlated with NADP⁺/NADPH with a correlation coefficient of 0.7 ($p < 0.001$). 5-Oxo-ETE was also positively correlated with the percent of glutathione present in the oxidized state (Fig. 10B) with a correlation coefficient of 0.65 ($p < 0.001$).

Discussion

Neutrophils maintained in culture underwent apoptosis, and by 24 h only 25% were annexin V/PI negative. This was associated with changes in the metabolism of AA, resulting in higher levels of the potent proinflammatory mediators 5-oxo-EETE and LTB₄. The increased amounts of LTB₄ were associated with reduced amounts of its metabolite 20-hydroxy-LTB₄, suggesting a reduced rate of ω -oxidation. In contrast, the increased levels of 5-oxo-EETE were accompanied by reduced amounts of its precursor 5-HETE, suggesting an increased rate of formation via 5-HEDH. These conclusions were substantiated by investigating the metabolism of 5-HETE and LTB₄ directly. Consistent with the data on AA metabolism, there was a dramatic shift in 5-HETE metabolism from ω -oxidation in freshly isolated neutrophils to 5-HEDH-catalyzed formation of 5-oxo-EETE in cells cultured for 24 h.

The altered 5-HETE and LTB₄ metabolism in cultured neutrophils could have been due to changes in either the amounts of 5-HEDH and LTB₄ 20-hydroxylase or their cofactors NADP⁺ and NADPH. However, when neutrophils were preincubated with PMS, which nonenzymatically converts intracellular NADPH to NADP⁺, the difference in 5-oxo-EETE synthesis between intact freshly isolated and cultured neutrophils was abolished, suggesting that total enzyme activity was unaltered, and this was confirmed by experiments with microsomal fractions from these cells. This suggests that the increased 5-oxo-EETE formation after 24 h was due to increased availability of NADP⁺. It was not possible to investigate 5-HEDH protein expression directly, as the enzyme has not yet been cloned and no antibodies are available. In contrast to 5-HEDH, microsomal LTB₄ 20-hydroxylase activity was significantly lower after 24 h, suggesting that the reduced metabolism of LTB₄ was due principally to a reduction in the amount of active enzyme.

To determine whether the changes in the formation of 5-LO products by neutrophils might be related to changes in pyridine nucleotides we measured the cellular levels of NAD⁺, NADP⁺, NADH, and NADPH. The 17-fold increase in 5-oxo-EETE formation in cultured neutrophils was accompanied by a similar 19-fold increase in NADP⁺ levels. However, NADPH, rather than declining as anticipated, was not significantly altered, and thus could not explain the decrease in ω -oxidation activity that we observed. The elevated NADP⁺ levels could potentially be due to the high levels of NAD⁺, which is converted to NADP⁺ by NAD kinase. This is the only pathway for the formation of NADP⁺ and it is required to maintain NADP⁺ and NADPH levels in cells [30]. It is activated following depletion of NADPH in bacteria and is important for maintaining the levels of this pyridine nucleotide under conditions of oxidative stress [31].

We examined the possibility that the increase in NAD⁺ was the driving force behind the altered 5-HETE metabolism in cultured neutrophils by blocking its formation with the nicotinamide phosphoribosyltransferase inhibitor FK866, which has been shown to deplete NAD⁺ in HepG2 human liver carcinoma cells [27]. Although this inhibitor dramatically lowered the levels of all four pyridine nucleotides in neutrophils, it had no effect on the metabolism of 5-HETE. This is presumably because although the levels of NADP⁺ and NADPH were both lowered by about 75%, their ratio was unaltered. We have previously shown that NADPH is a potent inhibitor of 5-oxo-EETE formation, and that the ratio of NADP⁺/NADPH is more important than the absolute concentration of NADP⁺ in regulating 5-HEDH activity [25]. We conclude from these experiments that under the conditions we employed, NAD⁺ levels are an important determinant of the total amount of NADP(H) in neutrophils, but have little effect on the NADP⁺/NADPH ratio, and consequently are not responsible for the altered 5-HETE metabolism in aging cells. Consistent with this interpretation, when the data from all of the conditions investigated in this study were examined together, 5-oxo-EETE synthesis was

positively correlated with the ratio of NADP⁺/NADPH ($r = 0.70$; $p < 0.001$) but not with NAD⁺ content ($r = 0.18$) (data not shown).

Because of its relationship with apoptosis [32] we investigated the involvement of oxidative stress by measuring glutathione redox status. In agreement with data in the literature for other cell types [33], we found that GSH is depleted in neutrophils after 24 h. In contrast, GSSG levels rose significantly during this period, resulting in a substantial increase in the ratio of GSSG/GSH, consistent with increased oxidative stress. Examination of the data from all of the conditions investigated revealed that cell survival was positively correlated with GSH content ($r = 0.82$) and negatively correlated with the percent of GSH that was oxidized ($r = 0.81$) (data not shown). However, the increased GSSG levels could not nearly account for the substantial depletion of GSH, which may be mediated by transport proteins, as shown for lymphoid cells [34], rather than the result of oxidation due to oxidative stress. Glutathione depletion has previously been shown to be required for apoptosis in lymphoid cells [34], and may compromise the ability of cells to inactivate ROS. Depletion of GSH and other thiols in neutrophils with diethylmaleate and diamide increased the rate of apoptosis [35], whereas augmentation of intracellular GSH levels had the opposite effect [36].

To further investigate the relationship between apoptosis and 5-HETE metabolism we examined the effects of agents that are known to promote neutrophil survival. The potent neutrophil survival factor GM-CSF [29] partially reversed the changes in 5-oxo-EETE and 5,20-diHETE formation, lowered the ratio of NADP⁺/NADPH, and inhibited GSH oxidation. Forskolin, which promotes neutrophil survival by activating adenylyl cyclase [28], had effects similar to GM-CSF on 5-oxo-EETE synthesis, and NADP⁺/NADPH, but did not increase 5,20-diHETE formation or lower GSSG levels. The lack of an effect of forskolin on the redox status of GSH suggests that cell survival can be increased without an appreciable effect on cellular antioxidant defence systems. Furthermore, the failure of forskolin to increase ω -oxidation activity suggests that its reduction in aging neutrophils may be due primarily to oxidative stress rather than to apoptosis *per se*. The different profile of responses to forskolin and GM-CSF is presumably related to their different signalling mechanisms.

To provide further evidence for a role of oxidative stress in the altered profile of 5-HETE metabolites and pyridine nucleotides in aging neutrophils we examined the effects of antioxidants that act at different levels (Fig. 11). As NOX2 is an important source of ROS in neutrophils, we investigated the effects of the NOX inhibitor DPI. DPI mimicked the effects of GM-CSF on 5-oxo-EETE formation and NADP⁺/NADPH, but had no effect on the extent of apoptosis after 24 h. The lowering of NADP⁺ levels by DPI suggests that NOX2 is active in generating ROS during the progression of neutrophils to apoptosis and is likely to contribute to the marked increase in NADP⁺ levels. However, NOX2 and ROS play a complex role in apoptosis, as NOX2-derived ROS inhibit caspase activity [37]. Apoptosis induced by PMA or during phagocytosis is accompanied by a robust activation of NOX2 and proceeds by an alternate pathway that is not dependent on caspase activation [38]. On the other hand, spontaneous apoptosis in neutrophils is accompanied by caspase activation [37], implying a diminished production of ROS in this process. Nevertheless, NOX2-derived ROS still appear to play a role in spontaneous apoptosis, as it is retarded in neutrophils from subjects with chronic granulomatous disease (CGD) [39]. This may be due to ROS-induced activation of ligand-independent death receptor signalling involving activation of acid sphingomyelinase [40]. The lack of effect of DPI on apoptosis may be the result of these opposing effects of ROS combined with the accelerated depletion of GSH that we observed, the mechanism for which is presently unclear. In addition, DPI has NOX2-independent effects such as inhibition of glucose 6-phosphate dehydrogenase, which would reduce the activity of the antioxidant pentose phosphate pathway [41].

NOX2-derived superoxide is reduced to H_2O_2 by SOD. However, we found that SOD did not affect cell survival or 5-HETE metabolism and had little or no effect on pyridine nucleotide or glutathione levels, suggesting that extracellular superoxide is not involved in these changes. However, as superoxide cannot diffuse freely through the cell membrane, we cannot exclude the involvement of intracellularly generated superoxide. Varied results have been reported for the effect of SOD on neutrophil apoptosis. Whereas one study suggested that it inhibits apoptosis in neutrophils [42], other groups either failed to show such an effect [39,43], as in the present study, or demonstrated accelerated apoptosis [44].

In contrast to SOD, we found that catalase has a pronounced inhibitory effect on neutrophil apoptosis, in agreement with other studies [39,43], and partially reversed all of the other changes we observed in aging neutrophils, strongly implicating H_2O_2 in these responses. H_2O_2 could act via the GSH (and/or thioredoxin) redox cycle, resulting in the glutathione peroxidase-catalyzed formation of GSSG and its reduction back to GSH by glutathione reductase, resulting in the generation of NADP^+ (Fig. 11). Alternatively, H_2O_2 could modify the activities of various intracellular signalling molecules and other proteins [45] or could be converted via the Fenton reaction to highly reactive hydroxyl radicals ($\text{HO}^\bullet$) (Fig. 11), which could affect a variety of cellular processes and compromise cellular antioxidant defences.

Deferoxamine had effects very similar to catalase, further supporting the involvement of ROS. Deferoxamine is an iron chelator and acts principally by inhibiting iron-catalyzed free radical reactions such as the Fenton reaction, but it is also a free radical scavenger and can thereby inactivate both $\text{HO}^\bullet$ and superoxide [46]. In agreement with previous studies [40,43], we found that it increases neutrophil survival. We also found that it inhibits aging-induced changes in 5-HETE metabolism, pyridine nucleotide levels, and cellular redox status, suggesting the involvement in these processes of $\text{HO}^\bullet$ or lipid peroxides, which, like H_2O_2 , are substrates for glutathione peroxidase.

Conclusions

As neutrophils age in culture they undergo a dramatic shift in 5-HETE metabolism from the biologically inactive ω -oxidation product 5,20-diHETE to the proinflammatory mediator 5-oxo-ETE. Furthermore, because of a reduction in its rate of metabolism, LTB_4 persists for a longer time, which would prolong its proinflammatory effects. The increased rate of 5-oxo-ETE formation is due to a dramatic increase in intracellular NADP^+ levels, resulting in an elevated ratio of $\text{NADP}^+/\text{NADPH}$. In spite of this, NADPH is not reduced in aging neutrophils, presumably due to the large increase in NAD^+ , which can be converted to NADPH by the successive actions of NAD kinase and glucose 6-phosphate dehydrogenase. Maintenance of NADPH levels in this way could help to explain the ability of aging neutrophils to undergo a robust respiratory burst when stimulated with PMA [47]. In contrast to 5-HEDH, the activity of which is undiminished over 24 h, LTB_4 20-hydroxylase activity declines, probably due to oxidative damage to the enzyme, which would explain the reduced formation of ω -oxidation products of LTB_4 and 5-HETE. For these changes in eicosanoid metabolism to manifest themselves *in vivo*, neutrophils may have to evade rapid engulfment by macrophages, which normally occurs as soon as phosphatidylserine shifts to the outer surface of the plasma membrane, and is associated with the resolution of inflammation [48]. However, neutrophils may persist under conditions of severe inflammation and proceed to late apoptosis and secondary necrosis when the phagocytic capacity of resident macrophages is exceeded [49], as has been shown to occur in a model of LPS-induced pulmonary inflammation [50]. This could be the case in diseases characterized by severe inflammation such as COPD (chronic obstructive pulmonary disease) and severe asthma, which are associated with persistently high levels of pulmonary neutrophils [2], as well as reperfusion injury following myocardial infarction and sepsis [51]. Under such conditions, 5-oxo-ETE and LTB_4 levels could be

elevated, leading to further infiltration of inflammatory cells and prolongation of inflammation. Preliminary studies in our laboratory suggest that other types of cells may also display an increased ability to synthesize 5-oxo-ETE when they are dying, and it is possible that this may contribute to the stimulatory effect of dying cells on inflammation [52].

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Fig. 1.

5-Lipoxygenase pathway in human neutrophils. Abbreviations: AA, arachidonic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; 5-oxo-20-HETE, 5-oxo-20-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HpETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5,20-diHETE, 5,20-dihydroxy-6,8,11,14-eicosatetraenoic acid; LT, leukotriene; 20h-LTB₄, 20-hydroxy-LTB₄; 5-LO, 5-lipoxygenase; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; LTA-h, leukotriene A₄ hydrolase.

Fig. 2.

Formation of 5-LO metabolites in A23187-stimulated neutrophils. Neutrophils (1×10^6 cells in 1 ml) suspended in RPMI containing 0.5% FBS were stimulated by addition of A23187 (5 μ M) and incubated in 6-well plates for up to 24 h. 5-HETE ($\circ$), 5-oxo-EETE ($\bullet$), and LTB₄ (Δ) were measured by RP-HPLC as described under Experimental Procedures.

Fig. 3.

Metabolism of AA by freshly isolated and cultured neutrophils. Freshly isolated neutrophils (A,C) or neutrophils that had been cultured for 24 h in RPMI/10% FBS (B,D) were suspended in PBS and incubated for 2 h (A,B) or various additional times (C,D) with AA (20 μ M) and A23187 (5 μ M). The products were measured by RP-HPLC as described in Materials and Methods (A,B) using a gradient prepared from solvents A (water), B (acetonitrile) and C (methanol), all containing 0.02% acetic acid, as follows: 0 min: 60% A, 30% B, 10% C; 42 min: 10% A, 38% B, 52% C. The flow rate was 1 ml/min. PGB₂ (100 ng) was used as an internal standard. The upper and lower tracings show absorbance at 235 and 280 nm, respectively. The time courses for the formation of 5-HETE (5h, $\circ$), 5-oxo-EETE (5o, $\bullet$), LTB₄ (B₄, $\blacktriangle$), and 20-

hydroxy-LTB₄ (hB₄, Δ) by freshly isolated neutrophils (**C**) and neutrophils cultured for 24 h (**D**) are also shown (n = 5). Other abbreviation in panels **A** and **B** are: dh, 5,20-diHETE; oh, 5-oxo-15-HETE; 15h, 15-HETE.

Fig. 4.

5-HETE metabolism by freshly isolated and cultured neutrophils. Freshly isolated neutrophils (**A**, **C**) or neutrophils that had been cultured for 24 h (**B**, **C**) (2×10^6 cells in 1 ml) were incubated with 5-HETE (4 μ M) for 30 min and the products measured by RP-HPLC as shown in panels **A** and **B**. The upper and lower tracings show the absorbance at 235 and 280 nm, respectively. The mobile phase was a gradient over 16.5 min between 35 and 75% acetonitrile in water, both containing 0.02% acetic acid, followed by 3 min of 75% acetonitrile under isocratic conditions. The flow rate was 1 ml/min. 13-HODE (13h, 100 ng) was used as an internal standard. **C**: The amounts of 5-oxo-EETE (5oETE) and 5,20-diHETE (diHETE) formed after incubation of 5-HETE with freshly isolated (open bars) or cultured (filled bars) neutrophils as described above for 30 min. ***, $p < 0.001$.

Fig. 5.

5-HEDH and ω -oxidation activity in aging neutrophils. **A:** Intact neutrophils (2×10^6 cells in 1 ml) were preincubated for 5 min at 37 °C with PMS(100 μ M) and then with 5-HETE for a further 30 min ($n = 23$). Alternatively, neutrophil microsomes (30 μ g protein in 1 ml) were incubated with either 5-HETE (4 μ M; **B**) or LTB₄ (4 μ M; **C**) for 10 min in the presence of either NADP⁺ (100 μ M) or NADPH (100 μ M), respectively. 5-HETE metabolites were measured by RP-HPLC as shown in Fig. 4 ($n = 5$). LTB₄ metabolites were also measured by RP-HPLC, using a gradient prepared from solvents A (water), B (acetonitrile) and C (methanol), all containing 0.02% acetic acid, as follows: 0 min: 60% A, 30% B, 10% C; 42 min: 10% A, 38% B, 52% C. The flow rate was 1 ml/min. Open bars: freshly isolated neutrophils; solid bars: neutrophils cultured for 24 h. *, $p < 0.05$.

Fig. 6.

Pyridine nucleotide levels in aging neutrophils. NADP⁺ and NAD⁺ were measured by RPHPLC in freshly isolated (**A**) and cultured (**B**) neutrophils as described under Experimental Procedures using deamino-NAD⁺ as an internal standard (IS). The small peak for NADP⁺ in panel A can easily be seen when the scale is expanded. **C**: NADP⁺ and NADPH and **D**: NAD⁺ and NADH levels in freshly isolated neutrophils (open bars) and neutrophils that had been cultured for 24 h (solid bars) (n = 14) were measured by RP-HPLC as shown in panels A and B (NAD(P)⁺) and as described under Experimental procedures (NAD(P)H). ***, p < 0.001.

Fig. 7.

Effects of FK866 on pyridine nucleotide levels and 5-HETE metabolism in cultured neutrophils. Neutrophils (2×10^6 cells in 1 ml) were cultured for 24 h in RPMI containing 10% FBS in the presence of either vehicle or FK866 (50 nM). Pyridine nucleotide levels ($n = 4$; **A**) and 5-oxo-EETE formation ($n = 6$; **B**) were measured by RP-HPLC as described under Experimental Procedures. In **B**, 5-HETE (4 μ M) was incubated for 30 min with neutrophils (2×10^6 cells in 1 ml) following preincubation with either vehicle (Veh) or PMS (100 μ M). 5-Oxo-EETE (5oETE) and 5,20-diHETE (5,20-dh) were then measured by RP-HPLC. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 8.

Effects of culturing neutrophils on survival and glutathione levels. Neutrophils were cultured for 24 h in RPMI 1640 containing 10% FBS. Survival was assessed staining freshly isolated neutrophils (**A**) and neutrophils that had been cultured for 24 h (**B**) with annexin V and propidium iodide followed by flow cytometry. The % survival in each of these groups (**C**) refers to the percent of cells that were not stained by either annexin V or PI (lower left quadrant in panels A and B; $n = 14$). The levels of GSH (**D**), 1/2GSSG (**E**), and the percent of total glutathione in the oxidized state (i.e. 1/2GSSG) (**F**) in neutrophils were determined by RP-HPLC as described in Experimental Procedures ($n = 12$). Open bars: freshly isolated neutrophils; solid bars: neutrophils cultured for 24 h. *, $p < 0.05$; ***, $p < 0.001$.

Fig. 9.

Effects of survival factors and antioxidants on 5-HETE metabolism, pyridine nucleotides, glutathione, and survival in neutrophils cultured for 24 h. All data are for neutrophils cultured for 24 h in the absence or presence of vehicle (Veh; n = 13–23, depending on the assay), 1 nM GM-CSF (GM; n = 4–10), 50 μM forskolin (Forsk; n = 5), 10 μM DPI (n = 5), 414 U/ml SOD (n = 5), 100 μg/ml catalase (Cat; n = 5–11), or 1 mM deferoxamine (Def; n = 4–6). The different parameters were measured as described under Experimental Procedures and the legends to the previous figures: survival (A; Fig. 8), 5-oxo-EETE (B) and 5,20-diHETE (C) in the presence of vehicle (Fig. 4), 5-oxo-EETE in the presence of 100 μM PMS (D; Fig. 5), the ratio of NADP⁺ to NADPH (E; Fig. 6), NAD⁺ (F; Fig. 6), GSH (G) and GSSG (H). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 10.

Correlation of 5-oxo-Ete formation with the ratio of NADP⁺/NADPH (A) and oxidized glutathione (B) in neutrophils. All of the individual values for the data from freshly isolated and cultured neutrophils, including all of the treatments included in Fig. 9, except for PMS-treated neutrophils, are shown. ½GSSG (%) refers to the percent of total glutathione that was present in the oxidized state.