Regulation of 5-hydroxyeicosanoid dehydrogenase activity in monocytic cells

Karl-Rudolf ERLEMANN^{*,1}, Chantal COSSETTE^{*}, Gail E. GRANT^{*}, Gue-Jae LEE[†], Pranav PATEL[†], Joshua ROKACH[†] and William S. POWELL^{*,2}

^{*}Meakins-Christie Laboratories, Department of Medicine, McGill University, 3626 St. Urbain Street, Montreal, Quebec H2X 2P2, Canada, and [†]Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Florida 32901-6982, U.S.A.

The 5-lipoxygenase product 5-oxo-ETE (5-oxo-eicosatetraenoic acid) is a highly potent granulocyte chemoattractant that is synthesized from 5-HETE (5-hydroxyeicosatetraenoic acid) by 5-HEDH (5-hydroxyeicosanoid dehydrogenase). In the present study, we found that 5-HEDH activity is induced in U937 monocytic cells by differentiation towards macrophages with PMA and in HL-60 myeloblastic cells by 1,25-dihydroxy-vitamin D₃. We used PMA-differentiated U937 cells to investigate further the regulation of 5-HEDH. This enzyme exhibits approx. 10 000-fold selectivity for NADP⁺ over NAD⁺ as a cofactor for the oxidation of 5-HETE, which is maximal at pH 10.2. In contrast, the reverse reaction (5-oxo-ETE → 5-HETE) is NADPH-dependent and is maximal at pH 6. Although the *K_m* for the forward reaction (670 nM) is about twice that for the reverse reaction at neutral pH, the *V_{max}* is approx 8-fold higher. The oxidation of 5-HETE to 5-oxo-ETE is supported by very low concentrations of NADP⁺ (*K_m*

139 nM), inhibited by NADPH (*K_i* 224 nM) and is consistent with a ping-pong mechanism. The amount of 5-oxo-ETE synthesized by 5-HEDH depends on the ratio of NADP⁺ to NADPH. Exposure of U937 cells to oxidative stress (t-butyl hydroperoxide) increased the ratio of NADP⁺ to NADPH from approx. 0.08 in resting cells to approx. 3, and this was accompanied by a dramatic increase in 5-HETE oxidation to 5-oxo-ETE. We conclude that differentiation of monocytic cells towards macrophages results in enhanced 5-oxo-ETE synthesis and that the ability of cells to synthesize 5-oxo-ETE is tightly regulated by the ratio of intracellular NADP⁺ to NADPH.

Key words: eicosanoid, 5-lipoxygenase pathway, monocyte, oxidative stress, 5-oxo-eicosatetraenoic acid (5-oxo-ETE), pyridine nucleotides.

INTRODUCTION

The 5-lipoxygenase pathway is responsible for the formation of a number of potent proinflammatory mediators that act via selective G-protein-coupled receptors. These include LTB₄ (leukotriene B₄), the cysteinyl-LTs (LTC₄ and LTD₄), 5-oxo-ETE (5-oxo-6, 8, 11, 14-eicosatetraenoic acid) and the lipoxins [1]. Among lipid mediators 5-oxo-ETE is the most active chemoattractant known for human eosinophils, eliciting a response 2–3 times higher than PAF (platelet-activating factor) and 30–40 times higher than LTB₄ and LTD₄ [2]. Furthermore, it has synergistic effects with PAF [2] and the CC chemokines eotaxin and RANTES (regulated upon activation, normal T-cell expressed and secreted) [3] in inducing eosinophil migration. 5-Oxo-ETE also induces a variety of other responses in these cells, including calcium mobilization, actin polymerization, expression of the adhesion molecule CD11b, shedding of L-selectin and degranulation [4–6]. It is active *in vivo*, eliciting pulmonary eosinophilia following intratracheal administration to Brown Norway rats [7] and infiltration of eosinophils and neutrophils into the skin in humans [8]. 5-Oxo-ETE also has chemoattractant effects on human neutrophils [9,10], and stimulates GM-CSF (granulocyte/macrophage colony-stimulating factor) release from the latter cells [11]. It acts through a highly selective G_i-protein-coupled receptor [the OXE (oxoeicosanoid) receptor] [12,13].

5-Oxo-ETE is synthesized from the 5-lipoxygenase product 5-HETE (5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid) by 5-HEDH (5-hydroxyeicosanoid dehydrogenase), which is present in leukocytes [14,15], platelets [16] and endothelial cells [17]. Relatively little is known about this enzyme, as it has not yet been cloned. It is localized in the microsomal fraction, prefers NADP⁺ as a cofactor and is highly selective for 5-HETE [14]. The availability of the cofactor NADP⁺ appears to be a limiting factor in the ability of cells to synthesize 5-oxo-ETE. Oxidative stress [18] and activation of the respiratory burst in phagocytes [19], both of which would elevate the intracellular levels of NADP⁺, stimulate 5-oxo-ETE synthesis strongly. This effect can be mimicked by PMS (phenazine methosulfate) [19], which converts intracellular NADPH to NADP⁺ [20].

The objective of the present study was to investigate the regulation of 5-HEDH. We first sought to determine whether this enzyme could be induced in myeloid cells, and found that PMA markedly up-regulates 5-HEDH activity in U937 cells. We then took advantage of the high 5-HEDH activity in differentiated U937 cells to investigate some of the properties of the enzyme and its regulation by cofactors. Measurement of pyridine nucleotides by HPLC revealed that oxidative stress induces changes in the intracellular levels of NADP⁺ and NADPH precisely in the range required to stimulate 5-oxo-ETE synthesis.

Abbreviations used: dh-VitD₃, 1,25-dihydroxy-vitamin D₃; FBS, foetal bovine serum; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; HEPSSO, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-hydroxypropanesulfonic acid); 5-HETE, 5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid; LT, leukotriene; ODS, octadecylsilyl; 5-oxo-ETE, 5-oxo-6, 8, 11, 14-eicosatetraenoic acid; PAF, platelet-activating factor; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase Type I; PGB₂, prostaglandin B₂; PMS, phenazine methosulfate; RA, retinoic acid; RP-HPLC, reversed-phase-HPLC; TBAH, tetrabutyl ammonium hydroxide; tBuOOH, t-butyl hydroperoxide.

¹ Present address: Charles River Laboratories Preclinical Services Montreal Inc., 22022 Trans-Canada Highway, Senneville (Montréal), Quebec H9X 3R3, Canada.

² To whom correspondence should be addressed (email William.Powell@McGill.ca).

MATERIALS AND METHODS

Materials and cell lines

5-HETE and 5-oxo-EETE [21] were prepared by total organic synthesis as described previously [22]. 5*R*-HETE was obtained from the Cayman Chemical Company. PMA, PMS, RA (retinoic acid), dh-VitD₃ (1,25-dihydroxy-vitamin D₃), PGB₂ (prostaglandin B₂), tBuOOH (t-butyl hydroperoxide), acetophenone, formic acid, NADPH, NAD⁺, deamino-NAD⁺, TBAH (tetrabutyl ammonium hydroxide), Mes, HEPSSO [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-hydroxypropanesulfonic acid)], Caps [3-(cyclohexylamino)propane-1-sulfonic acid], and CHAPS were purchased from Sigma-Aldrich. PMSF and DMSO were purchased from Fisher Scientific. NADP⁺ was from Roche Diagnostics. HL-60 and U937 cell lines were obtained from American Type Culture Collection. RPMI 1640 and other products used for cell culture were purchased from Invitrogen.

Cell culture

U937 and HL-60 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (foetal bovine serum). To induce differentiation, 2.5×10^5 cells/ml were cultured for up to 4 days with vehicle (0.2% DMSO), 50 nM dh-VitD₃, 1.3% (v/v) DMSO or 300 nM RA. In addition, 10^6 cells/ml were incubated with 18 nM PMA for the indicated times. The higher cell concentration used with PMA was necessary to ensure sufficient cell numbers, as it caused growth arrest. The cells remained in suspension for the duration of all of the above treatments except for PMA, which caused the cells to become adherent. In this case, the cells were resuspended by incubation with 2 mM EDTA on ice for 20 min in PBS⁻ (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄ and 8.1 mM Na₂HPO₄, pH 7.4), followed by scraping with a rubber policeman.

Preparation of microsomal fractions

Cells were washed by centrifugation at 200 *g* for 10 min at 4°C, 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) on ice for 5 × 6 s with 30 s intervals for cooling. The disrupted cells were centrifuged at 4°C at 1500 *g* for 10 min, 10 300 rev./min for 10 min and 41 000 rev./min in a Beckman 50.2T rotor for 80 min and the final pellet resuspended in PBS⁻. Protein was measured with the DC protein assay kit (Bio-Rad Laboratories).

Measurement of 5-oxo-EETE

To determine the capacity of intact cells to synthesize 5-oxo-EETE, 2.5×10^6 cells/ml were pre-incubated for 6 min with 100 μM PMS to convert intracellular NADPH into NADP⁺ and then incubated for 20 min at 37°C and pH 7.4 with 4 μM 5-HETE, unless otherwise indicated. Microsomal 5-HEDH activity was measured by incubation of microsomes (50 μg of protein/ml) with various concentrations of 5-HETE in the presence of 100 μM NADP⁺ for 20 min at 37°C and pH 7.4, unless otherwise indicated. All incubations were terminated by the addition of an equal volume of cold methanol (−20°C) and placed on ice. PGB₂ (125 ng) was added as an internal standard and the samples were stored at −80°C. Prior to analysis, the concentration of methanol was adjusted to 30% by the addition of water and the samples were centrifuged at 3300 *g* for 10 min at 4°C to remove any precipitated material. 5-Oxo-EETE was quantified by precolumn extraction/RP (reversed-phase)-HPLC [23] using a modified Waters Alliance system. The stationary phase was a column (150 mm × 3.9 mm) of ODS (octadecylsilyl)-silica (4 μm

particle size Novapak C₁₈ column; Waters Associates). The mobile phase was a linear gradient composed of solvents A [water/acetic acid (100:0.02, v/v)] and B [acetonitrile/acetic acid (100/0.02, v/v)] as follows: 0 min, 35% B; 10 min, 90% B.

Measurement of NADP⁺

NADP⁺ was measured by RP-HPLC following derivatization with alkaline acetophenone to give a fluorescent naphthyridine derivative (cf. [24]). U937 cells (2×10^6 cells in 250 μl) were incubated with tBuOOH for various times. The incubations were terminated by the addition of a mixture of 50 mM acetophenone and 3 M KOH along with 30 ng of deamino-NAD⁺ (as an internal standard) in 250 μl of 50% (v/v) methanol. After 20 min at 0°C, 62.5 μl of formic acid was added. After a further 15 min at 0°C, the mixture was extracted with ethyl acetate and the aqueous phase treated with 100 μM PMS. A 50 μl aliquot was then analysed by RP-HPLC on an Ultracarb ODS column (31% carbon loading; 5 μm particle size; 150 mm × 4.6 mm; Phenomenex). The mobile phase was a gradient between solvent C (100 mM citric acid containing 4 mM TBAH) and solvent D (acetonitrile) as follows: 0 min, 1% D; 12 min, 25% D; 14.3 min, 25% D). The flow rate was 1.25 ml/min. The NADP⁺-acetophenone adduct was detected using a Waters model 2475 Multiwavelength Fluorescence Detector (λ_{ex}, 371 nm; λ_{em}, 438 nm). The amounts of NADP⁺ were calculated from the ratios of the peak areas for NADP⁺ to those for the internal standard using a standard curve.

Measurement of NADPH

NADPH was measured by RP-HPLC, taking advantage of its native fluorescence. U937 cells (7.5×10^5 cells in 100 μl) were incubated for various times as described above for NADP⁺. The incubations were terminated by the addition of 200 mM Na₃PO₄. Aliquots (50 μl) from each of the samples were analysed by RP-HPLC using the ODS-silica column described above. The mobile phase was a gradient between solvents E (200 mM ammonium acetate containing 10 mM TBAH, adjusted to pH 6.0 with acetic acid) and F (methanol) as follows: 0 min, 3% F; 15 min, 17% F). NADPH was detected using a fluorescence detector (λ_{ex}, 325 nm; λ_{em}, 450 nm). The amounts of NADPH were calculated from a standard curve.

Data analysis

The effects of differentiating agents on HL-60 and U937 cells were compared using one-way repeated measures ANOVA with the Bonferroni test as a multiple comparison method. Paired *t* tests were used for the direct comparison of the *V*_{max} and *K*_m values of PMA and vehicle-treated U937 cells and for time courses. A *P* < 0.05 was considered significant. *n*, the number of independent experiments performed. The values shown are means ± S.E.M.

RESULTS

Induction of 5-HEDH by differentiating agents in U937 cells

U937 cells were cultured in the presence of either vehicle or a variety of agents known to induce cell differentiation, including PMA (18 nM), dh-VitD₃ (50 nM), RA (300 nM) and DMSO (1.3%). After 24 h in the presence of PMA the cells began to adhere to the surface of the wells, and were completely adherent by 2 days. None of the other agents induced adherence under the

Figure 1 Effects of various differentiation agents on 5-oxo-ETE synthesis by U937 cells

(A) U937 cells were cultured for 3 days (black bars) in the presence of vehicle (Veh), 18 nM PMA, 50 nM dh-VitD₃ (VitD), 1.3% DMSO or 0.3 μ M RA. Following pre-incubation with 100 μ M PMS for 6 min, the cells (2.5×10^6 /ml) were incubated with 4 μ M 5-HETE for 20 min at pH 7.4 and the amounts of 5-oxo-ETE determined by RP-HPLC. The amounts of 5-oxo-ETE produced by peripheral blood monocytes (Mono) and neutrophils (Neu) (hatched bars) are shown for comparison. (B) Time course for induction of 5-HEDH activity in U937 cells by PMA. U937 cells were treated with either vehicle (○) or 18 nM PMA (●). After different times the cells were harvested and 5-oxo-ETE formation was determined as described above. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n \geq 4$. (C) Lineweaver–Burk plots for the formation of 5-oxo-ETE by U937 cell microsomes. Microsomal fractions obtained from U937 cells treated with either vehicle (○) or PMA (18 nM) (●) for 4 days were incubated for 5 min at pH 7.4 with 100 μ M NADP⁺ and various concentrations of 5-HETE, after which time 5-oxo-ETE was measured by RP-HPLC. The results are one representative out of a total of five experiments with similar results (see Table 1).

Table 1 Lineweaver–Burk analysis of 5-HEDH activity in microsomal fractions from neutrophils and U937 cells

Microsomes (50 μ g of protein/ml) derived from U937 cells treated with either vehicle (control) or PMA (PMA-diff) for 4 days, or from circulating neutrophils, were incubated for 5 min at 37 °C with either fixed (4 μ M or 100 μ M respectively) or variable amounts of 5-oxo-ETE and NADP⁺ at pH 7.4. Alternatively, microsomal fractions (100 μ g of protein/ml) from PMA-differentiated U937 cells were incubated for 10 min with fixed (4 μ M or 100 μ M respectively) or variable amounts of 5-oxo-ETE and NADPH at pH 7.4. The amounts of 5-oxo-ETE or 5-HETE formed were determined by RP-HPLC and the K_m and V_{max} values analysed for each experiment using Lineweaver–Burk plots as shown in Figures 1(C), 4(A) and 6. ** V_{max} is significantly higher in microsomes from PMA-treated U937 cells compared with vehicle-treated controls ($P < 0.01$).

Source of microsomes	Reaction	Substrate (S) or inhibitor (I)	K_m or K_i (nM)	V_{max} [pmol/(min per μ g of protein)]	Number of independent experiments
Neutrophils	5-HETE $\rightarrow$ 5oETE	5-HETE (S)	399 ± 137	0.54 ± 0.30	4
Control U937	5-HETE $\rightarrow$ 5oETE	5-HETE (S)	568 ± 172	0.40 ± 0.12	5
PMA-diff U937	5-HETE $\rightarrow$ 5oETE	5-HETE (S)	670 ± 182	$1.29 \pm 0.19^{**}$	5
PMA-diff U937	5-oxo-ETE $\rightarrow$ 5-HETE	5-oxo-ETE (S)	274 ± 48	0.16 ± 0.01	6
PMA-diff U937	5-HETE $\rightarrow$ 5oETE	NADP ⁺ (S)	139 ± 15	1.09 ± 0.14	7
PMA-diff U937	5-oxo-ETE $\rightarrow$ 5-HETE	NADPH (S)	284 ± 79	0.23 ± 0.2	3
PMA-diff U937	5-HETE $\rightarrow$ 5oETE	NADPH (I)	224 ± 44		4

conditions employed. After 3 days in culture, the capacity of the cells (2.5×10^6 /ml) to synthesize 5-oxo-ETE was assessed following pre-incubation with 100 μ M PMS for 6 min, followed by incubation with 5-HETE (4 μ M) for a further 20 min and analysis of the products by RP-HPLC.

The effects of differentiating agents on 5-oxo-ETE synthesis are shown in Figure 1(A). PMA increased 5-oxo-ETE synthesis from 292 ± 31 pmol/10⁶ cells in vehicle-treated cells to 848 ± 100 pmol/10⁶ cells ($P < 0.001$), whereas DMSO and dh-VitD₃ induced approx. 2-fold increases in its synthesis ($P < 0.01$ and $P < 0.05$ respectively). RA tended to increase 5-oxo-ETE production, but this effect was not statistically significant. The amount of 5-oxo-ETE synthesized by vehicle-treated U937 cells was comparable with the amounts formed by peripheral monocytes and neutrophils (Figure 1A).

Effects of PMA on 5-HEDH activity in U937 cells

As PMA induced the greatest increase in 5-oxo-ETE synthesis, we examined the time course for this response. A significant effect on 5-oxo-ETE synthesis was observed by 2 days, when its rate of production was double that of control vehicle-treated cells ($P < 0.05$) (Figure 1B). The response to PMA reached a plateau be-

tween 3 and 4 days, when 5-oxo-ETE was synthesized at approx. 3 times the rate of cells treated with vehicle, which had no effect on 5-oxo-ETE synthesis.

To confirm that measurement of PMS-stimulated 5-oxo-ETE synthesis by intact cells is a good reflection of total 5-HEDH activity, we also assessed microsomal 5-HEDH activity. Microsomes (50 μ g of protein/ml) from cells that had been treated for 4 days with either PMA or vehicle were incubated with different concentrations of 5-HETE in the presence of 100 μ M NADP⁺ and the amounts of 5-oxo-ETE determined by RP-HPLC. Lineweaver–Burk analysis was used to determine K_m and V_{max} values (Figure 1C). PMA treatment resulted in an over 3-fold increase in the V_{max} ($P < 0.001$), but did not affect the K_m (Table 1). The K_m and V_{max} values for undifferentiated U937 cells were not significantly different from those for neutrophils (Table 1).

Induction of 5-HEDH activity in HL-60 cells by dh-VitD₃

The effects of the above differentiating agents on 5-oxo-ETE synthesis by HL-60 cells was also investigated (Figure 2A). Vehicle alone (0.1% DMSO) did not affect 5-oxo-ETE synthesis, which was similar to that observed for untreated U937 cells, monocytes and neutrophils. Of the agents tested, only dh-VitD₃ significantly

Figure 2 Effects of various agents on 5-oxo-EETE synthesis by HL-60 cells

(A) HL-60 cells were cultured for 3 days (black bars) in the presence of vehicle (Veh), 18 nM PMA, 50 nM dh-VitD₃ (VitD₃), 1.3% DMSO or 0.3 μ M RA, and the rates of 5-oxo-EETE synthesis were determined as described in the legend for Figure 1. The amounts of 5-oxo-EETE produced by peripheral blood monocytes (Mono) and neutrophils (Neu) (hatched bars) are shown for comparison. (B) Time course for the induction of 5-HEDH activity in HL-60 cells by dh-VitD₃. HL-60 cells were treated with either vehicle (○) or 50 nM dh-VitD₃ (●). After different times the cells were harvested and the rates of 5-oxo-EETE formation were determined as described in the legend for Figure 1. The values are means $\pm$ S.E.M. ($n \geq 4$). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

increased 5-oxo-EETE synthesis, which was over 2-fold higher than in vehicle-treated cells [715 ± 226 compared with 345 ± 50 pmol in 5-oxo-EETE/ 10^6 cells ($P < 0.001$)]. PMA induced only a modest change, which was not statistically significant, whereas RA and DMSO had no effect. In contrast with U937 cells, PMA did not induce adherence of HL-60 cells, but did result in the death of 20–50% of the cells by 4 days.

It has been reported that maximal neutrophilic differentiation of HL-60 cells with DMSO occurs after extended culture for up to 7 days [25,26]. In our hands, 1.3% (v/v) DMSO induced considerable loss of viability when cells were cultured in 10% (v/v) FBS. Over 40% were dead by day 4, as determined by their ability to exclude Trypan Blue. To investigate the potential effect of DMSO over longer periods, we grew HL-60 cells in 20% (v/v) FBS in the presence or absence of 1.3% (v/v) DMSO for up to 6 days [25,26]. At this time, control cells and DMSO-treated cells produced similar amounts of 5-oxo-EETE (363 ± 55 and 321 ± 25 nmol/ 10^6 cells respectively; $n = 3$; not significant).

The time course for the induction of 5-oxo-EETE synthesis by dh-VitD₃ in HL-60 cells is shown in Figure 2(B). 5-Oxo-EETE was significantly increased after 2 days ($P < 0.05$), with maximal rates of synthesis being observed after 3 days ($P < 0.01$). In contrast, there were no changes in 5-oxo-EETE synthesis by vehicle-treated cells over a period of 4 days.

Cofactor dependence for the oxidation of 5-HETE by 5-HEDH

In our original study reporting 5-HEDH activity in neutrophils [14], we found that incubation of microsomal fractions with 5-HETE in the presence of 1 mM NAD⁺ resulted in about one-third the amount of 5-oxo-EETE as was formed in the presence of 1 mM NADP⁺, suggesting that NAD⁺ might also support the oxidation of 5-HETE. To investigate the cofactor requirements for 5-HEDH in more detail, we incubated microsomal fractions from PMA-differentiated U937 cells with different concentrations of NAD⁺ and NADP⁺. As shown in Figure 3, near-maximal rates of formation of 5-oxo-EETE were achieved at low μ M concentrations of NADP⁺, whereas mM concentrations of NAD⁺ were required to observe significant formation of this compound. Assuming equivalent maximal responses, NADP⁺ was approx. 10 000 times more potent than NAD⁺ in supporting the oxidation of 5-HETE.

Figure 3 Effects of NADP⁺ and NAD⁺ on 5-HEDH activity in U937 cell microsomes

U937 cell microsomes (7.5 μ g of protein/ml) were incubated for 10 min at 37°C at pH 7.4 with 5-HETE (4 μ M) in the presence of different concentrations of NADP⁺ (●) or NAD⁺ (○). 5-Oxo-EETE was quantified by RP-HPLC. All values are means $\pm$ S.E.M. ($n = 4$).

Figure 4 Interconversion of 5-oxo-EETE and 5-HETE by U937 cells

(A) Lineweaver-Burk plots for the interconversion of 5-HETE (5h) and 5-oxo-EETE (5o). Different concentrations of 5-HETE were incubated with U937 cell microsomes (50 μ g of protein/ml) in the presence of NADP⁺ (100 μ M) for 5 min at pH 7.4 and 37°C and 5-oxo-EETE was quantified by RP-HPLC (5h $\rightarrow$ 5o; ●). Alternatively, different concentrations of 5-oxo-EETE were incubated with U937 cell microsomes (100 μ g of protein/ml) in the presence of NADPH (100 μ M) for 5 min at 37°C and 5-HETE was quantified (5o $\rightarrow$ 5h; ○). (B) Effects of pH on the interconversion of 5-HETE (5h) and 5-oxo-EETE (5o). U937 cell microsomes (50 μ g of protein/ml) in 50 mM Mes (circles), HEPSSO (triangles), or Caps (squares), adjusted to different pH values, were incubated for 5 min with either 1 μ M 5-HETE and 100 μ M NADP⁺ (closed symbols) or 1 μ M 5-oxo-EETE and 100 μ M NADPH (open symbols). The values are means $\pm$ S.E.M. ($n = 5$).

Stereoselectivity of 5-HEDH

To ensure that the 5-HEDH activity of U937 cells is similar to that present in neutrophils, and that it is not due to a non-selective mechanism, we investigated the stereoselectivity of the enzyme. At high substrate concentrations (4 μ M), the amount of 5-oxo-EETE formed from 5*R*-HETE was only $1.4 \pm 0.5\%$ of the amount formed from 5*S*-HETE (results not shown).

Reversibility of 5-HEDH

We previously observed that neutrophil microsomes convert 5-oxo-EETE stereoselectively into 5*S*-HETE [14]. To compare the kinetics of the forward and reverse reactions we measured the amounts of products formed after incubation of different concentrations of 5-HETE or 5-oxo-EETE with microsomal fractions from PMA-differentiated U937 cells in the presence of either NADP⁺ or NADPH respectively. Although the K_m of the reverse reaction was about one-half that of the forward reaction, the V_{max} of the forward reaction (5-HETE $\rightarrow$ 5-oxo-EETE) was about 8 times greater than that of the reverse reaction (Figure 4A and Table 1).

Figure 5 Mechanism for oxidation of 5-oxo-ETE by 5-HEDH

Different concentrations of 5-HETE were incubated with a microsomal fraction from U937 cells (50 μ g of protein/ml) for 5 min at 37°C at pH 7.4 in the presence of different concentrations of NADP^+ . (A) Lineweaver–Burk plots for the oxidation of 5-HETE by U937 cell microsomes in the presence of 0.1 μ M (●), 0.17 μ M (○), 0.3 μ M (■), 0.6 μ M (□), 1.5 μ M (▼) and 10 μ M (▽) NADP^+ . (B) The slope of each of the curves shown in (A) against the reciprocal of the concentration of NADP^+ . (C) The intercept (i.e. $1/V_{\max}$ for 5-HETE $\rightarrow$ 5-oxo-ETE) for each of the curves shown in (A) against the reciprocal of the concentration of NADP^+ . Data are for a single representative of seven independent experiments with similar results.

Effect of pH on the interconversion of 5-HETE and 5-oxo-ETE

The effects of pH on both the forward and reverse reactions catalysed by 5-HEDH were examined. Microsomal fractions from PMA-differentiated U937 cells were incubated with either 5-HETE or 5-oxo-ETE (1 μ M) along with the appropriate cofactor (100 μ M NADP^+ or 100 μ M NADPH respectively) for 5 min in 50 mM Mes, 50 mM HEPSSO or 50 mM Caps (Figure 4B), which permitted us to examine a pH range between 4 and 11.4. The forward and reverse reactions displayed quite distinct pH dependencies. The optimal pH for the forward reaction (5-HETE $\rightarrow$ 5-oxo-ETE) was observed at pH 10.2, whereas the reverse reaction (5-oxo-ETE $\rightarrow$ 5-HETE) was pH 6. The maximal rate for the forward reaction [2013 ± 273 pmol of 5-oxo-ETE/(min per mg)] was over 8 times greater than that [250 ± 56 pmol of 5-HETE/(min per mg)] for the reverse reaction. At physiological pH (pH 7.45), the rate of the forward reaction was more than double that of the reverse reaction. No oxidation of 5-HETE by NADP^+ alone, in the absence of microsomes, was observed at any of the pH values investigated.

Reaction mechanism of 5-HEDH

The formation of 5-oxo-ETE is a Bi Bi reaction, as it involves two substrates (5-HETE and NADP^+) and two products (5-oxo-ETE and NADPH). To determine whether this reaction follows a sequential or a ping-pong mechanism, we conducted experiments in which a series of concentrations of 5-HETE were incubated with U937 cell microsomes in the presence of different fixed concentrations of NADP^+ . The data from these experiments were analysed using Lineweaver–Burk plots (Figure 5A). Each line represents a series of concentrations of 5-HETE at one fixed concentration of NADP^+ . If the reaction follows a ping-pong mechanism (where the first product is released from the enzyme before the second substrate binds), the plots for different NADP^+ concentrations should be parallel to one another. On the other hand, if the reaction proceeds by a sequential mechanism (both substrates must bind to the enzyme before any products are released), the lines representing different NADP^+ concentrations should inter-

Figure 6 Lineweaver–Burk plot for NADPH

U937 cell microsomes (100 μ g of protein/ml) were incubated with 5-oxo-ETE (3 μ M) in the presence of different concentrations of NADPH for 10 min at 37°C at pH 7.4 and the amount of 5-HETE formed was measured by HPLC. The data are for a single representative of three separate experiments with similar results.

sect somewhere to the left of the ordinate. The representative experiment shown in Figure 5(A) reveals a series of approximately parallel lines with similar slopes, which would be consistent with a ping-pong mechanism. A graph of the slopes of these lines against $1/\text{NADP}^+$ (Figure 5B) reveals that there is no correlation between these parameters (i.e. the slope is close to 0), supporting their lack of convergence. This was supported by averaging the slopes of the ‘slope against $1/\text{NADP}^+$ ’ plots from all seven independent experiments, which gave a slope indistinguishable from 0 (0.008 ± 0.026). In contrast, there is a strong correlation between the apparent $V_{\max}$ value calculated for 5-HETE and the concentration of NADP^+ employed, as shown in the plot of $1/V_{\max}$ against $1/\text{NADP}^+$ (Figure 5C). The average value for the K_m for NADP^+ for all seven experiments was 139 ± 15 nM and the $V_{\max}$ was 1.09 ± 0.14 pmol of $\text{NADPH}/(\text{min per } \mu\text{g of protein})$.

Inhibition of 5-HEDH-catalysed oxidation of 5-HETE by NADPH

As described above, NADPH is required for the reduction of 5-oxo-ETE to 5-HETE by 5-HEDH. The approximate K_m and $V_{\max}$ values for NADPH were determined by incubating microsomal fractions from PMA-differentiated U937 cells with a saturating concentration of 5-oxo-ETE in the presence of different concentrations of NADPH . The rate of formation of NADP^+ was assumed to be identical with that of 5-HETE, as these two products would be formed in a 1:1 ratio. Lineweaver–Burk analysis of the data (Figure 6) gave a K_m value for NADPH of 284 ± 79 nM and a $V_{\max}$ of 0.23 ± 0.2 pmol/(min per μ g of protein) (Table 1).

The high affinity of NADPH for 5-HEDH suggested that it might inhibit the formation of 5-oxo-ETE from 5-HETE. To investigate this possibility we incubated a saturating concentration of 5-HETE (5 μ M) and different concentrations of NADP^+ with microsomes from PMA-differentiated U937 cells in the presence of increasing concentrations of NADPH . At the lowest concentration of NADP^+ tested (3 μ M), NADPH was a potent inhibitor of 5-oxo-ETE formation, with an IC_{50} value of 4.0 ± 0.7 μ M (results not shown). At higher NADP^+ concentrations, the curve for the inhibitory effect of NADPH was shifted to the right, giving IC_{50} values of 14 ± 1 μ M and 50 ± 5 μ M at concentrations of 10 and 30 μ M NADP^+ , respectively. When the amounts of 5-oxo-ETE formed in the above experiment were plotted against the ratio of NADP^+ to NADPH , it became clear that the ratio of these two cofactors was much more important than the absolute

Figure 7 Inhibition of 5-oxo-EETE formation by NADPH

Microsomal fractions (7.5 μg of protein/ml) from U937 cells were incubated with 5-HETE (4 μM) in the presence of different concentrations of NADP⁺ and NADPH for 10 min at 37 °C at pH 7.4. The amounts of 5-oxo-EETE formed were measured by RP-HPLC. (A) Inhibitory effects of different concentrations of NADPH in the presence of 3 (●), 10 (▽) or 30 μM NADP⁺. The amounts of 5-oxo-EETE are shown as percentages of the maximal amounts formed in the absence of NADPH and have been plotted against the ratios of NADP⁺ to NADPH. The values are means $\pm$ S.E.M. ($n=3$). (B) Lineweaver-Burk plots for NADP⁺ in the presence of different concentrations of vehicle (Veh; ●), 3 μM NADPH (△), or 10 μM NADPH (▲). The data are for a single representation of four separate experiments with similar results.

concentrations of either of them in regulating the oxidation of 5-HETE to 5-oxo-EETE (Figure 7A). The curves obtained for decreasing concentrations of NADPH in the presence of the three fixed concentrations of NADP⁺ were nearly superimposable.

The K_i for NADPH was determined by Lineweaver-Burk analysis, as shown in Figure 7(B). U937 cell microsomes were incubated with 5-HETE (4 μM) in the presence of different concentrations of NADP⁺ in the presence of vehicle, 3 μM NADPH, or 10 μM NADPH. The plots obtained in the presence of increasing concentrations of NADPH converged at the y-axis, indicating competitive inhibition. The K_i for NADPH was calculated from the x-intercepts to be 224 ± 44 nM.

Relationship between 5-oxo-EETE synthesis and intracellular levels of pyridine nucleotides

To relate our data on the effects of NADP⁺ and NADPH on 5-HEDH activity in microsomes to the regulation of 5-oxo-EETE syn-

thesis in intact cells, we compared the rate of 5-oxo-EETE synthesis to NADP⁺ and NADPH levels following stimulation of U937 cells with tBuOOH (100 μM). To ensure that our results were not affected by non-enzymatic oxidation of NADPH into NADP⁺ after the incubations were terminated, controls were performed in which tBuOOH was added immediately after the stopping solution. The values for NADP⁺ and NADPH in these samples were not significantly different from the values obtained for control cells not subjected to oxidative stress.

As shown in Figure 8(A), tBuOOH induced a rapid increase in 5-oxo-EETE synthesis within the first 5–10 min after its addition, with little further increase after 10 min. Similarly NADP⁺ rose dramatically by nearly 8-fold within 10 s of addition of tBuOOH, remained at about this level for 1.5 min and then began to decline, reaching basal levels by 20 min (Figure 8B). In contrast, NADPH levels declined precipitously within 10 s of addition of tBuOOH and then returned to normal within 20 min. The ratio of NADP⁺ to NADPH in resting cells was 0.082, which increased by 36-fold to a maximum value of 2.94, which was observed 10–30 s after the addition of tBuOOH, and then declined to 0.078 by 20 min (Figure 8C). The average rates of formation of 5-oxo-EETE within different time periods following addition of tBuOOH are also shown in Figure 8(C). The highest rates of formation were observed within the first 4 min (~ 2.5 pmol/min per 10^6 cells), after which time the rates declined to reach low levels by 20 min.

The approximate intracellular concentrations of NADP⁺ and NADPH in U937 cells were estimated using a cell volume of 911 fl as reported in the literature [27]. This gave basal levels of NADP⁺ and NADPH of 25 ± 2 and 306 ± 56 μM respectively, which changed to 194 ± 31 and 66 ± 18 μM respectively after the addition of tBuOOH. As these concentrations are somewhat higher than those used in the experiment with U937 cell microsomes illustrated in Figure 7(A), we conducted another experiment to determine the amounts of 5-oxo-EETE synthesized by microsomal fractions in the presence of pyridine nucleotides comparable with those found in resting and tBuOOH-stimulated cells. Changing the concentrations of NADP⁺ and NADPH from 25 and 300 μM respectively (cf. resting cells) to 200 and 65 μM (cf. stimulated cells) resulted in an increase in the rate of 5-oxo-EETE synthesis of about 6-fold (Figure 9). The relatively low concentration of NADPH found in stimulated cells had only a modest inhibitory effect on 5-oxo-EETE formation compared with the amount formed in the presence of NADP⁺ alone.

Figure 8 Effects of tBuOOH on 5-oxo-EETE synthesis and pyridine nucleotide levels in intact U937 cells

(A) U937 cells (10^6 cells/ml) were incubated with 5-HETE (1 μM) for different times at pH 7.4 in the presence of either vehicle (○) or 100 μM tBuOOH (●), and 5-oxo-EETE was measured by RP-HPLC. (B) U937 cells were incubated for different times in the presence of 100 μM tBuOOH, and NADP⁺ (●) and NADPH (△) were quantified by RP-HPLC as described in the Materials and methods section. (C) The ratios of NADP⁺ to NADPH (from B; ●) are plotted along with the average rates of formation of 5-oxo-EETE (○) over different time periods (from A) following the addition of tBuOOH to U937 cells. The values are means $\pm$ S.E.M. ($n=5$), except for the NADP⁺/NADPH ratios shown in (C), in which case S.E.M. could not be calculated because the NADP⁺ and NADPH measurements were not from the same cell preparations.

Figure 9 5-Oxo-ETE synthesis by U937 cell microsomes at physiological levels of pyridine nucleotides

5-HETE (4 µM) was incubated for 10 min at 37 °C and pH 7.4 with U937 cell microsomes (15 µg of protein/ml; $n = 3$) in the presence of concentrations of NADP⁺ and NADPH comparable with those found in resting (25 and 300 µM respectively) and tBuOOH-stimulated (200 and 65 µM respectively) U937 cells. 5-Oxo-ETE was quantified by RP-HPLC as described in the Materials and methods section.

DISCUSSION

We used U937 and HL-60 cells to investigate whether 5-HEDH activity could be induced during differentiation of myeloid cells. U937 cells are frequently used to study monocyte/macrophage differentiation, which can be induced by treatment with PMA [28], dh-VitD₃ [29], RA [30] or DMSO [31]. In contrast, these cells cannot be differentiated into granulocytes [32]. HL-60 cells, on the other hand, can be differentiated into monocyte/macrophage-like cells by dh-VitD₃ [33], into macrophage-like cells by PMA [34] and into granulocytic cells by RA [35] or DMSO [36]. The present study demonstrates that differentiation of both of these cell lines towards monocytes/macrophages results in enhanced 5-HEDH activity, whereas differentiation towards neutrophils (in the case of HL-60 cells) does not increase enzyme activity. The greatest degree of induction was observed when U937 cells were treated with PMA, which transformed them to adherent macrophage-like cells and increased their capacity to synthesize 5-oxo-ETE by over 3-fold, several times higher than that found in peripheral neutrophils and monocytes.

The inducibility of 5-HEDH in monocytic cells raises the possibility that this enzyme could be induced during the differentiation of peripheral monocytes into tissue macrophages. It has been reported previously that 5-oxo-ETE synthesis is increased in monocytes that have undergone differentiation into dendritic cells [37]. However, in this case, it was not clear whether this was due to increased enzyme activity or to alterations in intracellular cofactor levels. The release of 5-oxo-ETE by activated macrophages and dendritic cells, which are at the front line of host defence, could result in the tissue infiltration of granulocytes.

Other enzymes that act on eicosanoids have been reported to be induced in HL-60 cells. Neutrophilic differentiation with DMSO leads to the induction of LTA₄ hydrolase [38], which catalyses LTB₄ formation, along with low levels of LTB₄ 20-hydroxylase (CYP4F3A; [39]). Although the latter enzyme could potentially result in metabolism of 5-oxo-ETE [40], we did not observe any ω -oxidation products of either 5-HETE or 5-oxo-ETE in the present study, possibly owing to the relatively low expression levels of the hydroxylase and/or because LTB₄ is a better substrate for this enzyme than 5-oxo-ETE and 5-HETE. Relatively low levels of 5-lipoxygenase [41] and LTC₄ synthase [42] activity have been reported in HL-60 cells differentiated with DMSO or PMA. These agents also induce another eicosanoid dehydrogenase, 15-PGDH

(15-hydroxyprostaglandin dehydrogenase Type I), in these cells [43]. The induction of 5-HEDH in HL-60 cells contrasts with that of the other enzymes referred to above, as it is restricted to differentiation towards macrophages with dh-VitD₃ and is not observed following neutrophilic differentiation with DMSO. This might be indicative of a special role for the macrophage as a site of 5-oxo-ETE synthesis as mentioned above.

Neutrophil microsomes also catalyse the reverse reaction (5-oxo-ETE → 5S-HETE) in the presence of NADPH [14]. As shown in Figure 4(B), the interconversion of 5-HETE and 5-oxo-ETE by 5-HEDH is highly sensitive to pH, with the forward reaction (5-HETE → 5-oxo-ETE) being favoured at pH 6 and above, and the reverse reaction being favoured at pH 5. The rate of formation of 5-oxo-ETE increases dramatically at higher pH values, reaching a maximum at pH 10.2. Although both the forward and reverse reactions display pH optima well outside the physiological range, this is unlikely to have much impact on reaction rates in intact cells under physiological conditions. The effects of pH on 5-HEDH activity are similar to those on the activity of 15-PGDH, which displays maximal forward and reverse reaction rates at pH 8.8 (or above) and pH 5.5 respectively [44]. However, 15-PGDH differs substantially from 5-HEDH in other respects in that it is a cytosolic NAD⁺-dependent enzyme with substrate K_m values substantially higher than 5-HEDH [45]. Furthermore, because of the high intracellular levels of NAD⁺, 15-PGDH activity would not be limited by cofactor availability. The greater degree of regulation of 5-HEDH seems logical, as it is responsible for the synthesis of a biologically active product, whereas 15-PGDH is required for biological inactivation of prostaglandins.

The results of the present study show that the oxidation of 5-HETE to 5-oxo-ETE by 5-HEDH using NADP⁺ as the electron acceptor apparently follows a ping-pong mechanism. Although we cannot determine from our data whether 5-HETE or NADP⁺ binds first to the enzyme, it would seem likely that NADP⁺ first converts the enzyme into an oxidized state and then dissipates before 5-HETE binds. This is in contrast with 15-PGDH, which follows a sequential ordered mechanism [46].

Our previous results suggested that the intracellular level of NADP⁺ is a critical factor in regulating 5-oxo-ETE production [18,19]. However, the very low K_m (approx. 140 nM) that we found for NADP⁺ in the present study would suggest that even very low concentrations of this cofactor would be sufficient to support 5-oxo-ETE formation. This raised the possibility that the regulation of 5-HEDH activity might be more complex, possibly involving other factors such as NADPH. As illustrated in Figure 7, NADPH is a potent inhibitor of 5-oxo-ETE formation, with a K_i of 224 µM. Thus in the presence of NADPH, much higher levels of NADP⁺ are required to permit substantial formation of 5-oxo-ETE.

To put these findings in a physiological perspective, we measured the levels of NADP⁺ and NADPH in U937 cells under basal and stimulated conditions. We have shown previously that resting U937 cells synthesize only relatively small amounts of 5-oxo-ETE, whereas cells stimulated with hydrogen peroxide or tBuOOH synthesize much larger amounts [18]. This effect could be blocked by inhibitors of glutathione reductase, suggesting that the effect of peroxides is mediated by their glutathione peroxide-induced reduction coupled to oxidation of GSH, and recycling of the resultant GSSG back to GSH by glutathione reductase, thus generating NADP⁺. Further evidence for this hypothesis was obtained in the present study by direct measurement of NADP⁺ and NADPH. The ratio of NADP⁺ to NADPH was only about 0.08 in unstimulated cells, but rose within 10 s to nearly 3 following treatment with tBuOOH.

As the absolute amounts of NADP⁺ and NADPH that we measured were higher than those used in our initial microsomal experiments (cf. Figure 7A), we investigated the effects of physiological concentrations of these cofactors on 5-oxo-ETE formation by U937 cell microsomes. Conditions mimicking resting cells (low NADP⁺, high NADPH) resulted in a low rate of synthesis, whereas conditions similar to those in cells subjected to oxidative stress (high NADP⁺, low NADPH), resulted in a much higher rate of synthesis. Thus it would appear likely that, within the physiological range of pyridine nucleotide concentrations, the ratio of NADP⁺ to NADPH is more important in determining the rate of 5-oxo-ETE synthesis than the absolute concentrations of either of these cofactors. The ratios of NADP⁺ to NADPH in resting and stimulated cells are indicated by the dotted lines added to the graph shown in Figure 7(A), illustrating the inhibitory effect of NADPH on 5-oxo-ETE synthesis by U937 cell microsomes. It can be seen that the ratio in resting cells is at the bottom of the curve and would be expected to support only a low level of 5-oxo-ETE synthesis, whereas the ratio in stimulated cells is near the top of the curve. Thus the intracellular concentration ranges of NADP⁺ and NADPH correspond precisely to those that would be required to regulate 5-HEDH activity. Although the intracellular concentration of NAD⁺ is approx. 40 times higher than that of NADP⁺ in U937 cells (results not shown), NAD⁺ is unlikely to have much impact on 5-HEDH activity in intact cells, as it is over 10 000 times less active than NADP⁺ in supporting 5-HETE oxidation (Figure 3).

In conclusion, 5-HEDH is induced during differentiation of U937 cells and HL-60 cells towards monocytes/macrophages, suggesting that these cells may be an important site for 5-oxo-ETE synthesis in inflammation. 5-HEDH displays a high degree of selectivity for both 5S-HETE and NADP⁺, and follows a ping-pong Bi Bi mechanism. The formation of 5-oxo-ETE is tightly regulated by the ratio of NADP⁺ to NADPH within the cell and is suppressed by the high levels of NADPH found in resting cells. Oxidative stress, by dramatically increasing the ratio of NADP⁺ to NADPH, strongly stimulates 5-oxo-ETE formation. This may be an important mechanism in inflammation, which is accompanied by oxidative stress, which in turn would increase 5-oxo-ETE synthesis, resulting in further infiltration of inflammatory cells and prolongation of the inflammatory response. 5-HEDH or the 5-oxo-ETE receptor may therefore be attractive targets for novel anti-inflammatory drugs.

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