Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation

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The characteristics of hydroperoxide activation of 5-lipoxygenase were examined in the high speed supernatant fraction prepared from rat polymorphonuclear leukocytes. Stimulation of 5-lipoxygenase activity by the 5-hydroperoxyeicosatetraenoic acid (5-HPETE) reaction product was strongly dependent on the presence of thiol compounds. Various reducing agents such as mercaptoethanol and glutathione (0.5–2 mM) inhibited the reaction and increased the concentrations of 5-HPETE (1–10 μ M) necessary to achieve maximal arachidonic acid oxidation. The requirement for 5-HPETE was not specific and could be replaced by H_2O_2 (10 μ M) but not by the 5-hydroxyeicosatetraenoic acid (5-HETE) analogue. Furthermore, gel filtration chromatography of the soluble extract from leukocytes resolved different fractions which can increase the hydroperoxide dependence or fully replace the stimulation by 5-HPETE. Maximal activity of the 5-HPETE-stimulated reaction required Ca^{2+} ions (0.2–1 mM) and ATP with the elimination of the HPETE requirement at high ATP concentrations (2–4 mM). In addition, NADPH (1–2 mM), FAD (1 mM), Fe^{2+} ions (20–100 μ M) and chelated Fe^{3+} (0.1 mM-EDTA/0.1 mM- FeCl_3) all markedly increased product formation by 5-lipoxygenase whereas NADH (1 mM) was inhibitory and Fe^{3+} (20–100 μ M) alone had no effect on the reaction. The stimulation by Fe^{2+} ions and NADPH was also observed under various conditions which increase the hydroperoxide dependence such as pretreatment of the enzyme preparation with glutathione peroxidase or chemical reduction with 0.015% NaBH_4 . These results provide evidence for an hydroperoxide activation of 5-lipoxygenase which is not product-specific and is modulated by thiol levels and several soluble components of the leukocytes. They also indicate that stimulation of 5-lipoxygenase activity can contribute to increase lipid peroxidation in iron and nucleotide-promoted reactions.

INTRODUCTION

The availability of lipid hydroperoxides is generally considered to play an important regulatory role in the biosynthesis of eicosanoids derived from arachidonic acid. Several studies have demonstrated that HPETEs stimulate the biosynthesis of leukotrienes (Kanaji *et al.*, 1986; Maclouf *et al.*, 1982, 1986) and prostaglandins (Hemler *et al.*, 1978; Kulmacz & Lands, 1983) and thus may be implicated in the pathophysiology of a wide range of diseases including atherosclerosis and asthma (Bray, 1986; Warso & Lands, 1984).

The characterization of lipoxygenase and cyclooxygenase activities in cell-free systems also indicated that the enzyme reactions are sensitive to activation by their hydroperoxide products and analogues (Hemler & Lands, 1980; Rouzer & Samuelsson, 1986). While product activation of the enzymes provides a simple explanation to account for the stimulatory effects of HPETEs on arachidonic acid oxidation, more studies on the availability of hydroperoxides and on the mechanism of activation are required to evaluate the contribution of hydroperoxides in the regulation of eicosanoid biosynthesis.

The first step in the oxidation of arachidonic acid to leukotrienes is catalysed by 5-lipoxygenase, producing the highly unstable epoxide leukotriene A_4 in a reaction

that involves 5-HPETE as intermediate (Hogaboom *et al.*, 1986; Shimizu *et al.*, 1986; Ueda *et al.*, 1986). The activity of 5-lipoxygenase has also been shown to be stimulated by HPETEs in some cases, although the level of stimulation varies. A marked stimulation of 5-lipoxygenase activity by 5-HPETE was observed for the human (Rouzer & Samuelsson, 1986) and rat (Riendeau & Leblanc, 1986) leukocyte enzymes (20- and 4-fold, respectively), whereas only a reduction in the duration of the initial lag phase of the reaction was detected for the murine mast cell enzyme (Bryant *et al.*, 1986). Furthermore, 5-lipoxygenases from porcine leukocytes and rat basophilic leukaemia cells have recently been purified to apparent homogeneity with high yields (15–50%) without the use of HPETEs to measure activity (Hogaboom *et al.*, 1986; Ueda *et al.*, 1986). This variability may indicate that differences in experimental conditions and enzyme purity could affect the level of HPETE dependence, although these aspects have not been investigated.

Studies on the mechanism of activation of lipoxygenases by hydroperoxides using the soybean enzyme have shown that these compounds oxidize the non-haem iron of the protein to the ferric form (Schewe *et al.*, 1986). Recent experiments show that ferric lipoxygenase can easily be inactivated by reduction, suggesting that changes in the oxidation state of the enzyme may be

Abbreviations used: 5,12-DiHETE, 5,12-dihydro-6,8,10,14-eicosatetraenoic acid; DTT, dithiothreitol; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 13-HPOD, 13-hydroperoxyoctadecadienoic acid.

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important for the expression *in vivo* of lipoxygenase activity (Kemal *et al.*, 1987). This aspect of the regulation of 5-lipoxygenase was investigated in the present study by characterizing the hydroperoxide requirement for the enzyme reaction in the soluble fraction from rat polymorphonuclear leukocytes. The hydroperoxide stimulation was found to be strongly dependent on the presence of thiols and other stimulatory and inhibitory endogenous factors from leukocyte lysates. Furthermore, experiments performed under limiting conditions of hydroperoxides permitted us to demonstrate a nucleotide- and iron-dependent stimulation of 5-lipoxygenase capable of replacing product activation of the enzyme.

A preliminary report of this work has been published in abstract form at the 14th International Congress of Biochemistry, Prague, July 1988.

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid and [14 C]arachidonic acid (390 mCi/mmol) were obtained from Sigma Chemical Co. and New England Nuclear, respectively. FAD, NAD(P)H and ATP (crystalline, vanadium-free, 99–100%) were from Sigma Chemical Co. Ultrogel AcA 34 was purchased from LKB. 5(*S*)-HETE and racemic 5-HPETE were synthesized as previously described (Zamboni & Rokach, 1983) and were a gift from Y. Leblanc (Merck Frosst). Protein concentrations were measured using the Bio-Rad dye-binding assay.

Enzyme preparation

Polymorphonuclear leukocytes were prepared from rat peritoneal exudates collected 18–20 h after injection of sodium caseinate (Cunningham *et al.*, 1979). Contaminating red blood cells were lysed by resuspending the pelleted cells in deionized water for 15 s at room temperature (5 ml/50 ml of exudate), followed by the addition of an equivalent volume of 1.8% NaCl. The leukocytes were then pelleted by centrifugation and lysed at 4 °C by sonication (3 × 30 s pulses at 70% duty cycle and output 3 using a Cole Parmer Ultrasonic homogenizer) at a concentration of 2×10^8 cells/ml in 10 mM-Hepes (Na⁺ salt), pH 7.3, containing 2 mM-EDTA and 1 mM-2-mercaptoethanol. The soluble fraction (100 000 g supernatant) was prepared from the sonicated cells as previously described (Riendeau & Leblanc, 1986) and contained 3–6 mg of protein/ml.

Assay of 5-lipoxygenase

The activity of 5-lipoxygenase was measured from the conversion of [14 C]arachidonic acid to 5-HETE and 5,12-diHETEs using t.l.c. to resolve the products of the reaction (Ochi *et al.*, 1983; Soberman *et al.*, 1985). The standard incubation mixture contained 25 mM-sodium/potassium phosphate buffer, pH 7.3, 1 mM-ATP, 0.5 mM-CaCl₂, 0.5 or 2.5 mM-mercaptoethanol and 12–25 μ l of the enzyme preparation in a final volume of 0.2 ml. The enzyme was preincubated at 37 °C for 2 min before initiation of the reaction with the addition of 2 μ l of [14 C]arachidonic acid (25 000 d.p.m.) in ethanol to obtain a final concentration of 10 μ M (Riendeau & Leblanc, 1986). Fatty acid hydroperoxides (5-HPETE or 13-HPOD) were added with the arachidonic acid solution to obtain the indicated final concentrations. After incu-

bation for 10 min at 37 °C, the reaction was stopped by adding 0.8 ml of diethyl ether/methanol/1 M-citric acid (30:4:1, by vol.). The samples were centrifuged at 1000 g for 5 min and the organic phases were dried under N₂, redissolved in 100 μ l of diethyl ether and analysed by t.l.c. on Baker Si250F-PA or Whatman LKGF plates using diethyl ether/light petroleum/acetic acid (50:50:1, by vol.) as solvent. The amount of radioactivity migrating at the positions of arachidonic acid, 5-HETE and 5,12-diHETEs was determined using a Berthold t.l.c. analyser LB 2842. 5-HETE was the major reaction product (ratio HETE/diHETE > 4) and only traces of other lipoxygenase products were detected, in agreement with previous observations (Skoog *et al.*, 1986a). The 5-lipoxygenase activity is reported as the percentage of conversion of arachidonic acid to 5-HETE and 5,12-diHETEs after the 10 min incubation (plateau levels). Less than 4% of background radioactivity was detected at the positions of products using heat-denatured enzyme. The percentage of arachidonic acid oxidized was dependent on enzyme concentration over the range of 0.2–1.0 mg of protein/ml and the curves obtained under different assay conditions are shown in Fig. 1. Under conditions where no HPETE or mercaptoethanol was added, 30% arachidonic acid conversion corresponded to an activity of 7 nmol of product per mg of protein. The results were confirmed using two to five different enzyme preparations.

RESULTS

Dependence of the HPETE stimulation on reducing agents

Previous experiments have shown that fatty acid hydroperoxides are required to obtain high levels of 5-lipoxygenase activity for the thiol-stabilized enzyme (Rouzer & Samuelsson, 1986). The relationship between reducing conditions and hydroperoxide levels was further investigated by determining the effect of mercaptoethanol and 5-HPETE on 5-lipoxygenase activity using different amounts of protein (Fig. 1). The enzyme activity was measured from maximal product formation (plateau level) which increased linearly with protein concentration up to 0.5–1 mg/ml in the absence of exogenous reducing agent and HPETE (Fig. 1a). Addition of 5-HPETE at a concentration of 10 μ M decreased the oxidation of arachidonic acid by about 0.4 nmol/10 min (Fig. 1a). A completely different picture was observed when the assays were performed in the presence of reducing agents. Fig. 1(b) shows that mercaptoethanol at a concentration of 2.5 mM strongly inhibited 5-lipoxygenase at all protein concentrations. The addition of 10 μ M-5-HPETE markedly stimulated the activity in this case, to a level of activity comparable to that observed with 5-HPETE in the absence of reducing agent. The stimulation of 5-lipoxygenase activity by 5-HPETE in the presence of mercaptoethanol was a consistent observation for 10 different enzyme preparations. However, the level of inhibition by 10 μ M-5-HPETE in the absence of mercaptoethanol was variable (corresponding to 0–0.5 nmol of arachidonic acid oxidized/10 min, depending on the preparation).

The level of inhibition of 5-lipoxygenase progressively increased with the concentration of mercaptoethanol, reaching 50% at 0.5–1 mM. In contrast, no effect by this reducing agent up to a concentration of 5 mM could be

Fig. 1. Dependence of 5-lipoxygenase activity on protein concentration

The activity was measured using different amounts of the 100000 g supernatant in the absence (■) or in the presence (●) of 10 µM-5-HPETE. (a) Incubation mixtures without reducing agent; (b) incubation mixtures containing 2.5 mM-mercaptoethanol.

Fig. 2. Variation in the 5-HPETE dependence of 5-lipoxygenase activity according to reducing conditions

Enzyme activity was measured in the presence of varying amounts of 5-HPETE using incubation mixtures containing no reducing agent (■), 0.5 mM-mercaptoethanol (●) or 2.5 mM-mercaptoethanol (▲).

detected in the presence of 10 µM-5-HPETE (results not shown). However, the concentration of 5-HPETE required to obtain maximal arachidonic acid oxidation by 5-lipoxygenase was found to increase with the concentration of reducing agent. For example, Fig. 2 shows

Fig. 3. Peroxide requirement for the stimulation of 5-lipoxygenase activity

5-Lipoxygenase activity was measured as described under 'Experimental procedures' except that 5-HETE and hydroperoxides were added at the end of the preincubation period (2 µl of a 1 mM solution to obtain 10 µM final concentrations), immediately before arachidonic acid addition. The reactions were performed in the absence (-SH) or presence (+SH) of 2.5 mM-mercaptoethanol. The amount of 5-lipoxygenase reaction products was calculated from the conversion of arachidonic acid to 5-HETE and 5,12-diHETEs for assays run in triplicate ($\pm$ S.E.M.).

the addition of 1 µM-5-HPETE was sufficient to stimulate the reaction to near maximal levels when the assay buffer contained 0.5 mM-mercaptoethanol, while 5–10 µM-5-HPETE was required to obtain a similar activity with 2.5 mM-mercaptoethanol. Product formation in reactions stimulated by the hydroperoxide reached the same level as that measured in the absence of thiol at 5–10 µM-HPETE. These results indicate that the 5-HPETE requirement for 5-lipoxygenase activity is increased by reducing conditions.

Specificity of the peroxide stimulation

The specificity of the 5-HPETE stimulation was determined by substitution of this compound by the corresponding alcohol, 5-HETE, and by H₂O₂ (Fig. 3). In the absence of added thiol, 5-HETE caused an inhibition similar to that produced by 5-HPETE at a concentration of 10 µM (47% and 65% respectively) whereas H₂O₂ had no significant effect. 5-HETE addition did not increase the activity of the enzyme inhibited by mercaptoethanol, indicating that the hydroperoxide group of the eicosatetraenoic acid is required for stimulation. In contrast, both H₂O₂ and HPETE abolished the inhibition by the reducing agent and increased enzyme activity to levels comparable to those measured in the absence of mercaptoethanol (Fig. 3). The stimulation of 5-lipoxygenase activity by hydroperoxides in soluble extracts is thus not restricted to product or product analogues of the reaction. The data from Figs. 1 and 3 suggest that 5-HPETE has a dual effect on 5-lipoxygenase activity, i.e. (1) the ability to reverse the inhibition by thiols which requires the presence of the hydroperoxide group, and (2) an inhibitory effect which appears to be related to the fatty acyl chain moiety since it was also observed with 5-HETE but not with H₂O₂.

Table 1. Decrease in inhibitory effects of reducing agents on 5-lipoxygenase activity by 5-HPETE

5-Lipoxygenase activity was measured as described under 'Experimental procedures' in the absence or the presence of the indicated reducing agent. Each value is the average of two to four determinations. Results are given as percentages of the maximal activity obtained in the absence of reducing agent and HPETE (1.0 nmol of arachidonic acid oxidized/10 min).

Reducing agent	Concentration (mM)	5-Lipoxygenase activity (%)	
		No 5-HPETE	10 μ M-5-HPETE
None	—	100	62
Mercaptoethanol	0.1	107	52
	2.5	< 5	52
Dithiothreitol	0.1	< 5	68
	2.5	< 5	43
Glutathione	0.1	< 5	46
	2.5	< 5	20

Selectivity of the inhibition by reducing agents

The inhibition of 5-lipoxygenase activity was observed in the presence of different reducing agents, such as glutathione or dithiothreitol (Table 1). The results at low concentrations (0.1 mM) indicated that glutathione and DTT were more potent inhibitors than mercaptoethanol. As described above for mercaptoethanol, the addition of 10 μ M-5-HPETE also restored a significant proportion of enzyme activity in the presence of inhibitory concentrations of these reducing agents (Table 1).

Replacement of HPETE stimulation by endogenous factors

To investigate in more detail the contribution of endogenous components from the cell extracts on the 5-HPETE dependence of 5-lipoxygenase activity, the supernatant fraction was concentrated 3-fold and subjected to gel filtration chromatography. The chromatography was performed without either phospholipid or ethylene glycol necessary for recovery of 5-lipoxygenase activity (Goetze *et al.*, 1985; Hogaboom *et al.*, 1986), in order to increase resolution and dissociation of components possibly interacting with the enzyme. No lipoxygenase activity was thus detected after chromatography under these conditions (results not shown). However, the eluting fractions strongly modified the HPETE dependence of the 5-lipoxygenase activity of the 100000 g supernatant. Fig. 4 shows the profile of an Ultrogel AcA 34 column (resolution M_r 20000–350000) where the eluting fractions were assayed in the presence of a constant amount of the supernatant without 5-HPETE addition. A stimulatory peak of 5-lipoxygenase activity was eluted after the void volume (fractions 33–36) and was followed by inhibitory fractions (fractions 37–40) and a second peak of stimulatory fractions (fractions 41–43). Similar experiments with gel filtration on Ultrogel AcA 44 also resolved one stimulatory peak from an inhibitory peak (results not shown). In contrast, no stimulation or inhibition of 5-lipoxygenase activity by

Fig. 4. Gel filtration of stimulatory and inhibitory factors of 5-lipoxygenase activity

The soluble fraction from leukocyte lysates (5.6 ml) was concentrated 3-fold using Centricon-30 filters (Amicon), applied on an Ultrogel AcA 34 column (118 cm $\times$ 1 cm) and eluted at 4 $^{\circ}$ C in 0.05 M-sodium/potassium phosphate, pH 7.3, containing 0.1 mM-mercaptoethanol. 5-Lipoxygenase activity (■) was measured in the presence of 0.5 mM-mercaptoethanol and without hydroperoxide addition using 40 μ l from the elution fractions (1.6 ml total volume) mixed with 15 μ l of the initial supernatant fraction. The elution fractions had no detectable 5-lipoxygenase activity and had no effect on the enzyme activity of the supernatant in the presence of 5-HPETE (results not shown). The protein content of the fractions is given (○) and the level of 5-lipoxygenase activity of the supernatant fraction (no elution fraction added) is shown by the horizontal line (23% conversion). The positions of bovine serum albumin (M_r 67000) and chymotrypsinogen A (M_r 25000) are indicated by the numbers 1 and 2, respectively. Similar results were obtained when the soluble fraction was not concentrated before gel filtration although the level of stimulation of 5-lipoxygenase activity by the eluting fractions was less pronounced in this case (1.5–1.7-fold).

these fractions could be detected when the assays were performed in the presence of 5-HPETE, even when the amount of supernatant was reduced to obtain a basal level of 20% conversion of arachidonic acid (results not shown).

These experiments indicate that 5-lipoxygenase activity is dependent on the concentration of various endogenous components of the supernatant fraction and that 5-HPETE stimulation masks their effects. They also provide evidence for the presence of factor(s) in leukocyte lysates which can replace the stimulation of lipoxygenase activity by hydroperoxides.

ATP and calcium requirements

ATP and calcium ions have been reported to stimulate 5-lipoxygenase activity from various sources (Bryant *et al.*, 1986; Ochi *et al.*, 1983; Rouzer & Samuelsson, 1985), including the rat enzyme (Furukawa *et al.*, 1984; Skoog *et al.*, 1986a). This was also the case for the 5-HPETE-stimulated 5-lipoxygenase for which maximal activity was observed in the presence of 0.3–1 mM-calcium (Fig. 5a) and ATP at concentrations higher than 0.25 mM (Fig. 5b). Interestingly, ATP also eliminated the inhibitory effect of mercaptoethanol at high concentrations (1 and 4 mM-ATP to achieve the same level of product

Fig. 5. Effect of calcium and ATP on the 5-HPETE stimulation of 5-lipoxygenase activity

Enzyme activity was measured in the presence of 2.5 mM-mercaptoethanol with (●) or without (■) the addition of $10 \mu\text{M}$ -5-HPETE as described under 'Experimental procedures'. (a) Incubation markers contained a fixed amount of ATP (1 mM) and the indicated concentrations of CaCl_2 ; (b) incubation mixtures contained CaCl_2 (0.5 mM) and the indicated concentrations of ATP.

Table 2. Stimulation of 5-lipoxygenase activity by adenine cofactors

5-Lipoxygenase activity was measured using a fixed amount of the 100 000 g supernatant from rat polymorphonuclear leukocytes in the presence of 0.5 mM-mercaptoethanol. Cofactors and 13-HPOD were added at final concentrations of 1 mM and $1 \mu\text{M}$, respectively. Results are the average of two to five determinations $\pm$ S.E.M. obtained with the same enzyme preparation. Similar results were obtained with three different enzyme preparations using mercaptoethanol concentrations ranging from 0.5 to 2.5 mM.

Addition	Arachidonic acid oxidized (%)
None	12 ± 3 (n = 4)
NADPH	27 ± 6 (n = 5)
NADP ⁺	20 ± 6 (n = 3)
NADH	< 4 (n = 2)
NAD ⁺	< 4 (n = 2)
FAD	35 ± 10 (n = 3)
13-HPOD	33 ± 6 (n = 4)

formation as with $10 \mu\text{M}$ -5-HPETE using two different preparations). The effect of ATP might be explained by a protection of the enzyme against mercaptoethanol inhibition or by the generation of activating peroxides.

Replacement of the hydroperoxide stimulation by adenine dinucleotide cofactors

The replacement of the 5-HPETE stimulation by ATP suggests that this nucleotide might promote reactions leading to the generation of hydroperoxide. To investigate this possibility further, a series of experiments were performed to examine the effects of conditions known to promote lipid peroxidation. The activity of 5-lipoxygenase was measured in the presence of 2-mercaptoethanol (0.5–2.5 mM) in order to introduce a marked dependence on hydroperoxides which was routinely evaluated using 13-HPOD ($1 \mu\text{M}$) as positive control. Under these conditions, FAD, NADPH and NADP⁺ all increased arachidonic acid oxidation by 5-lipoxygenase at a concentration of 1 mM (Table 2). The effect of NAD(P)H was specific since the reaction could not be detected in the presence of either NADH or NAD⁺ at the same concentration. The stimulation was observed only in the absence of an exogenous source of hydroperoxide, these enzyme cofactors being without significant effect on product formation in the presence of $1 \mu\text{M}$ -13-HPOD (results not shown).

Stimulation of the 5-lipoxygenase reaction by iron

Both the oxidation state and the concentration of iron are important factors in the promotion of lipid peroxidation in biological systems (Aust & Svingen, 1982). Fig. 6 shows that the hydroperoxide requirement for 5-lipoxygenase activity in the presence of thiol could be replaced by the addition of iron or an iron-chelate complex. Concentrations of Fe^{2+} of $20 \mu\text{M}$ and above stimulated the oxidation of arachidonic acid by 5-lipoxygenase to levels comparable to those obtained with 13-HPOD. This effect was specific for the reduced form

Fig. 6. Stimulation of 5-lipoxygenase by Fe^{2+}

The percentage conversion of arachidonic acid to 5-HETE and leukotriene B_4 by the 100 000 g supernatant fraction was measured after 10 min in the presence of 0.5 mM-mercaptoethanol and the indicated concentrations of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (○) or FeCl_3 (●). In the absence of iron, the conversion was stimulated from 8% to 28% by the addition of $1 \mu\text{M}$ -13-HPOD under identical assay conditions.

Table 3. Stimulation of 5-lipoxygenase activity by Fe^{2+} and NADPH after pretreatment with NaBH_4

Aliquots from the 100000 *g* supernatant fraction were preincubated with the assay mixture at 4 °C for 20 min in the presence or absence of 0.015% NaBH_4 . NADPH or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were then added and the mixture incubated for 2 min at 37 °C prior to the initiation of the reaction with arachidonic acid ($\pm$ 13-HPOD). The reaction was stopped after a 5 min incubation at 37 °C and the percentage of conversion to 5-lipoxygenase reaction products was determined by t.l.c. Results are averages of duplicates.

NaBH_4	Addition	Arachidonic acid oxidized (%)
—	—	33
+	—	< 3
+	13-HPOD (1 μM)	19
+	NADPH (1 mM)	20
+	Fe^{2+} (50 μM)	17

of iron, with Fe^{3+} having little effect over the same range of concentrations (Fig. 6). However, the stimulation by 1 μM -13-HPOD was completely replaced by chelated complex 0.1 mM-EDTA/0.1 mM- Fe^{3+} and 0.2 mM-ADP/0.1 mM- Fe^{3+} (results not shown).

In all experiments where a stimulation was observed, 5-HETE was the major product (> 80%) with lower amount of 5,12-diHETEs derived from the leukotriene A_4 product of 5-lipoxygenase. Furthermore, reactions could not be detected using heat-inactivated enzyme or incubations in the absence of calcium and ATP (< 4% arachidonic acid oxidized), confirming that the observed stimulations were due to 5-lipoxygenase activity and not to a non-enzymic oxidation of arachidonic acid. The time-dependence of arachidonic acid oxidation by NADH- and Fe^{2+} -stimulated reactions corresponded to that obtained with 1 μM -13-HPOD, with maximal product formation being reached after a few minutes ($t_{1/2}$ = 0.4–0.5 min) (results not shown).

Effect of Fe^{2+} and NADPH under limiting conditions of hydroperoxide activation

In the experiments described in Fig. 6 and Table 2, incubations were carried out in the presence of reducing agents to achieve a maximal dependence of the 5-lipoxygenase reaction on exogenous hydroperoxides. Therefore, the effects of Fe^{2+} and NADPH on the 5-lipoxygenase reaction were also measured after pretreatment of the enzyme preparation with NaBH_4 or glutathione peroxidase in order to increase the hydroperoxide requirement without the use of inhibitory thiols. For the experiment described in Table 3, NaBH_4 was used to reduce chemically endogenous hydroperoxides from the enzyme preparation. The concentration of borohydride and incubation times were adjusted to reduce the oxidation of arachidonic acid by 5-lipoxygenase to near background levels with preservation of the stimulation by 13-HPOD. Under these conditions, the presence of either 50 μM - Fe^{2+} or 1 mM-NADPH caused a marked stimulation of the formation of 5-lipoxygenase reaction products (Table 3). The amount of

Table 4. Effect of NADPH and Fe^{2+} on the inhibition of 5-lipoxygenase activity by glutathione-peroxidase

Identical volumes of the 100000 *g* supernatant were incubated at 37 °C for 1 min in the absence (control) or in the presence of 0.75 unit of glutathione peroxidase (GP)/ml. NADPH or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was then added and the mixtures were incubated at 37 °C for another 2 min before the addition of arachidonic acid ($\pm$ 13-HPOD). The amount of 5-lipoxygenase reaction product was determined after 3 min for reactions performed in duplicate.

Conditions	Arachidonic acid oxidized (%)
Control	42
+ GP	4.4
+ GP + NADPH (2 mM)	11
+ GP + Fe^{2+} (50 μM)	30
+ GP + 13-HPOD (1 μM)	47

product formed in these cases was comparable to that obtained with 13-HPOD (17–20% conversion).

Similar experiments were performed using glutathione peroxidase to introduce a 10-fold stimulation by 13-HPOD (Table 4). The addition of 50 μM - Fe^{2+} increased product formation by about 7-fold whereas NADPH caused only a low level of stimulation (2.5-fold at 2 mM). In other experiments, we have noticed that Fe^{2+} was also more effective than NADPH in restoring 5-lipoxygenase activity at high NaBH_4 concentrations (results not shown). Fe^{3+} did not stimulate the activity at 50 μM . These results indicate that Fe^{2+} and NADPH can stimulate the 5-lipoxygenase activity under several different conditions which make the reaction dependent on hydroperoxides.

DISCUSSION

Modulation of the HPETE activation of 5-lipoxygenase by thiols and leukocyte factors

The present results demonstrate that the 5-lipoxygenase activity of rat polymorphonuclear leukocytes is sensitive to inhibition by thiols and that the inhibited enzyme can be stimulated by hydroperoxides. The high dependence of peroxide stimulation on the presence of reducing agents is in agreement with previous observations on the human and rat enzymes where incubation mixtures containing DTT and mercaptoethanol were used (Riendeau & Leblanc, 1986; Rouzer & Samuelsson, 1986). In addition, our results show that the amount of 5-HPETE required to activate the enzyme is directly related to the concentration of thiol and that maximal product formation by 5-lipoxygenase could be obtained in the absence of 5-HPETE when reducing agents were also omitted from the reaction mixture. It has been shown that lipid peroxidation occurs more rapidly in extracts than in healthy tissues (Halliwell & Gutteridge, 1984) and presumably endogenous peroxides are present in the supernatant preparation or generated during incubation in amounts sufficient to stimulate 5-lipoxygenase maximally in the absence of exogenous reducing agent. In this case, increasing the hydroperoxide level with 5-HPETE (e.g. 10 μM) resulted in inhibition of

the reaction whereas an identical amount of 5-HPETE would be stimulatory in the presence of thiol.

The inhibition of 5-lipoxygenase by thiols could be either due to an inactivation of the enzyme or to the elimination of endogenous peroxides. DTT and other antioxidants have recently been shown to inactivate soybean lipoxygenase by reduction of the non-haem ferric ion of the enzyme to the ferrous form (Kemal *et al.*, 1987). Such a mechanism could explain 5-lipoxygenase inhibition and the increased requirement for hydroperoxide to regenerate the active ferric form of the enzyme in the presence of thiols. Stimulation of peroxidase activity by reducing agents could also decrease the concentration of endogenous hydroperoxides to levels below those required to initiate the reaction. The observation that mercaptoethanol was a less potent inhibitor than DTT or glutathione is in agreement with the relative efficiency of these reducing agents in supporting 5-HPETE reduction in rat polymorphonuclear leukocyte extracts (Skoog *et al.*, 1986b). The concentrations of glutathione that caused inhibition of 5-lipoxygenase were in the same range as those found in tissues (0.1–1 mM) (Meister, 1988).

The results also provide evidence that several components of the supernatant fraction modulate the level of 5-lipoxygenase activity measured in the absence of exogenous peroxides. The detection of inhibitory fractions after gel filtration of leukocyte extracts may be explained by the presence of peroxidases (Skoog *et al.*, 1986b; Egan *et al.*, 1983), and this point was not investigated further. Of more interest is the identification of the two other fractions which stimulate 5-lipoxygenase activity in the absence of exogenous 5-HPETE. It is not known at the present time if the presence of two stimulatory peaks indicate distinct components or a broad peak superimposed with the inhibitory peak. Such stimulatory component(s) would probably not have been detected in similar mixing experiments with the human 5-lipoxygenase using assay mixtures containing 20 μ M-HPETE (Rouzer & Samuelsson, 1985), since the enzyme should be fully activated under these conditions. The importance of the role of these inhibitory and stimulatory factors in the modulation of 5-lipoxygenase activity in the 100000 *g* supernatant fraction is underlined by previous observations that the purified enzyme is less sensitive to inhibition by thiols and activation by HPETE (Goetze *et al.*, 1985; Ueda *et al.*, 1986). Recent experiments suggest that leukotriene synthesis in intact cells may also be regulated by the level of free thiols under certain circumstances, since the treatment of macrophages with *N*-ethylmaleimide (Peters-Golden & Shelly, 1987) and the depletion of intracellular glutathione of leukocytes (Hatzelmann & Ullrich, 1987) have been shown to increase the production of 5-lipoxygenase metabolites.

Replacement of product activation by H_2O_2 and conditions that promote peroxidation

The use of reducing conditions has allowed us to demonstrate that H_2O_2 and various cofactors which promote lipid peroxidation can also stimulate the 5-lipoxygenase reaction. The lack of specificity of the hydroperoxide requirement is in contrast with results obtained with human leukocyte supernatants where no stimulation of activity by H_2O_2 was observed (Rouzer & Samuelsson, 1986). Whether this discrepancy is due to

differences in methodology or enzyme specificities remains to be determined. H_2O_2 has been shown to increase the production of 5-HETE in rat lung (Burghuber *et al.*, 1985), suggesting that this peroxide may also stimulate 5-lipoxygenase activity in intact tissues. In contrast, high concentrations of H_2O_2 (e.g. 0.1 mM) have been reported to inhibit 5-lipoxygenase activity in macrophages (Sporn & Peters-Golden, 1988) and in sonified RBL-1 cells (Egan *et al.*, 1983).

The requirement of the 5-lipoxygenase reaction for hydroperoxides could be replaced by ATP, other nucleotide enzyme cofactors and iron. There is a striking similarity between the conditions which increase 5-lipoxygenase activity and those reported to promote enzymic lipid peroxidation *in vitro*: specificity for NADPH, effective concentration range of Fe^{2+} (20–100 μ M) and requirement of chelation for the effect of Fe^{3+} (Aust & Svingen, 1982; Kornbrust & Mavis, 1980; Wills, 1969). In microsomal preparations, NADPH:cytochrome *P*-450 reductase has been identified as the enzyme responsible for the promotion of NADPH/iron-dependent lipid peroxidation (Pederson *et al.*, 1973). A closely related NADPH:cytochrome *c* reductase/NADPH oxidase system is also present on the plasma membrane of polymorphonuclear leukocytes (Takayama *et al.*, 1984) which can also generate H_2O_2 by reduction of oxygen to superoxide anion followed by a dismutase reaction (Badwey & Karnovsky, 1980). This system requires both NADPH and FAD for maximal activity (Gabig & Babior, 1979). The 100000 *g* supernatant fraction from rat polymorphonuclear leukocytes contains some cytochrome *c* reductase activity (0.03 μ mol/min per mg of protein) and the stimulation of peroxidative reactions could then result in the activation of 5-lipoxygenase. The replacement of hydroperoxide activation by membrane-bound enzyme activities would also be in agreement with the observations that 5-lipoxygenase activity in subfractions containing membranes is relatively insensitive to inhibition by reducing agents (our unpublished work) and activation by 5-HPETE (Riendeau & Leblanc, 1986) and also with recent reports that 5-lipoxygenase translocates to the leukocyte membrane during ionophore challenge and leukotriene production (Rouzer & Kargman, 1988; Wong *et al.*, 1988).

In summary, we have shown that H_2O_2 and various promoters of lipid peroxidation can activate 5-lipoxygenase and that thiol levels and leukocyte components modulate the effect of hydroperoxides. These observations suggest that the leukotriene pathway may be activated by various reactions leading to hydroperoxide generation in addition to the lipoxygenase-derived HPETE products. They also provide a basis to examine the relationship between lipid peroxidation and leukotriene production in inflammation and ischaemic disorders, where both processes are believed to play an important role (Hammond *et al.*, 1985; Lefter, 1986).

We would like to thank B. Pearce for her excellent secretarial assistance.

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Received 23 March 1989/1 June 1989; accepted 30 June 1989