The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase

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Sulfhydryl reagents such as dithiothreitol stabilized human leukocyte 5-lipoxygenase (5-LO) during purification. During enzyme assay, however, these reagents led to irreproducible or unexpectedly low activity. This inconsistency in the assay was eliminated by inclusion of hydroperoxyeicosatetraenoic acids (1–5 μM) during the reaction which effected a 10–20-fold stimulation of 5-LO activity. Structural studies indicated that an intact hydroperoxy function, and a long-chain fatty acyl moiety were required for 5-LO stimulation. These data suggest that human leukocyte 5-LO is activated by hydroperoxy fatty acids, and that this results in a requirement for exogenous hydroperoxide in the presence of sulfhydryl reagents.

Lipoxygenase *Leukotriene* *Hydroperoxyeicosatetraenoic acid* *Hydroxyeicosatetraenoic acid* *Arachidonic acid* *Inflammation*

1. INTRODUCTION

The enzyme, 5-LO catalyzes the first two steps in the biosynthesis of leukotrienes from 20:4 [1]. Owing to the proposed role of leukotrienes in inflammation and immediate hypersensitivity, 5-LO has recently become the subject of intensive research [2]. In this laboratory work has been directed toward the purification of 5-LO from human leukocytes [3]. During the course of these studies we noted that the human leukocyte 5-LO requires activation by the product hydroperoxy fatty acid. In this report we present data on the concentration and structural dependence of the hydroperoxide stimulation. We also show how failure to recognize the hydroperoxide requirement (as has occurred in the past) can result in serious experimental error in the assay of 5-LO activity.

Abbreviations: 5-LO, 5-lipoxygenase; 20:4, arachidonic acid; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; GSH, glutathione

2. MATERIALS AND METHODS

2.1. *Leukocyte homogenates*

Human leukocyte homogenate 10000 × *g* and 100000 × *g* supernatants were prepared as described [3,4].

2.2. *Preparation of fatty acyl hydroperoxides*

A mixture of nonenzymatically generated HPETEs was prepared by the method of Boeynaems et al. [5]. 15-HPETE and 13-hydroperoxylinoleic acid were produced by the incubation soybean lipoxygenase with 20:4 and linoleic acid, respectively [6]. 12-HPETE was generated through the reaction of porcine leukocyte 12-lipoxygenase with 20:4 by the method of Yoshimoto et al. [7]. 5-HPETE was produced using 5-lipoxygenase prepared from potato tubers, incubated with 20:4 as described [8]. The 15-, 12- and 5-HPETEs were purified by silicic acid column chromatography [1,3] followed by straight-phase HPLC on a column (10 × 500 mm) of 10 μPolygosil silica (Machery-Nagel) eluted at 4 ml/min with hexane/isopropanol/

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acetic acid (99:1:0.01).

HPETEs were converted to their corresponding HETEs by reduction with $NaBH_4$ [6]. Methyl esters were formed by the reaction of fatty acids for 5 min with ethereal diazomethane. The concentrations of HPETE in solution were estimated by the absorbance at 235 nm ($\epsilon = 29500$). The identity and purity of HPETE solutions were verified by the co-chromatography with standards of the free acids and methyl esters of the reduced and unreduced compounds on straight- and reverse-phase HPLC systems.

2.3. *Enzyme assay*

For the preparation of assay substrates, the desired quantity of HPETE or HETE solution was evaporated to dryness under a stream of argon, and dissolved in a purified solution of [^{14}C]20:4 (20 mM, 66000 dpm/μl) to provide 5 μl per assay sample.

Cumene hydroperoxide and *t*-butyl hydroperoxide were obtained from Sigma. Solutions were prepared by diluting the commercial reagents in ethanol, to give the desired quantity of hydroperoxide in a total volume of 5 μl/sample. These solutions were then mixed 1:1 with the [^{14}C]20:4 substrate for use in the assay (10 μl/sample). Hydrogen peroxide was added as 5 μl of an aqueous solution immediately prior to the addition of substrate.

Details of the assay conditions and analysis of samples by silicic acid column chromatography and HPLC have been published [3]. One unit of 5-LO activity is the amount of enzyme producing 1 nmol of 5-HPETE under standard assay conditions.

2.4. *Protein assay*

Protein concentrations were estimated by the method of Bradford et al. [9] using bovine serum albumin as a standard. 10000 × *g* and 100000 × *g* supernatants of leukocyte homogenates contained 2.0–3.5 mg/ml protein.

3. RESULTS

3.1. *Stimulation of leukocyte 5-LO by HPETEs*

Initial studies on the 5-LO activity of crude human leukocyte homogenates indicated that inclusion of a sulfhydryl reagent such as DTT or GSH at a concentration of 1 mM helped to stabilize the enzyme during cell homogenization. However when enzyme assays were performed in the presence of these reagents we found that the activity of 10000 × *g* supernatants was highly erratic, and that of 100000 × *g* supernatants was unexpectedly low. Thus, in six separate experiments activities of 10000 × *g* supernatants were 1.20, 1.75, 2.82, 39.5, 56.8, and 61.0 units/ml, whereas the activities of 100000 × *g* supernatants were nearly always less than 5 units/ml.

The involvement of sulfhydryl reagents in this phenomenon led us to recall earlier work by Gibian and Galaway [10] and by Smith and Lands [11] that showed that the lipoxygenase from soybeans required its product hydroperoxy fatty acid as an activating factor, and that GSH peroxidase in the presence of GSH or DTT could indefinitely extend the kinetic lag phase in the reaction of this enzyme with linoleic acid. It seemed likely that DTT or GSH added to leukocyte homogenates could interact with endogenous peroxidases to produce a similar effect. If this were true, then addition of HPETEs to the reaction mixture should eliminate the effect of the sulfhydryl reagents, and produce a marked enzyme stimulation. This hypothesis was verified in experiments in which a leukocyte homogenate 10000 × *g* supernatant containing 1 mM DTT was assayed for 5-LO activity in the presence or absence of a mixture of nonenzymatically generated HPETEs. With purified 20:4 alone, a 5-LO activity of 0.5 units/ml was detected. Inclusion of 16–32 μM of the HPETEs resulted in an increase of the enzyme activity to 10.5 units/ml. No such stimulation was observed when the HPETEs were replaced by their corresponding HETEs.

3.2. *Comparison of the stimulatory activity of various fatty acyl hydroperoxides*

Fig.1 shows that 5-, 12-, and 15-HPETE as well as 13-hydroperoxylinoleic acid were all similar in their capacity to stimulate the human leukocyte 5-LO. All of these fatty acyl hydroperoxides were effective in the concentration range of 0.5–5 μM, and during the course of multiple experiments, no consistent, significant difference was observed in the activities of these different compounds. Interestingly, the HPETE methyl esters were similar-

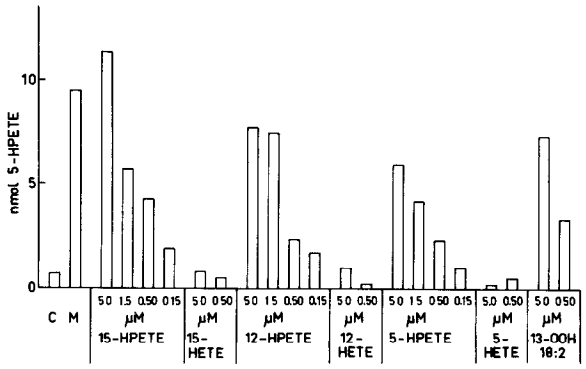

Fig.1. Structural requirement and concentration dependence of 5-lipoxygenase stimulation by fatty acyl hydroperoxides. Samples contained 300 μl samples of a leukocyte homogenate 100000 × *g* supernatant, prepared in the presence of 1 mM DTT, and were assayed under standard conditions with 100 μM [^{14}C]20:4 and the indicated concentrations of fatty acyl hydroperoxides or hydroxides. The bar labeled C represents 5-HPETE production in the absence of any stimulating factor. The sample labeled M received 20 μM mixed nonenzymatically generated HPETEs.

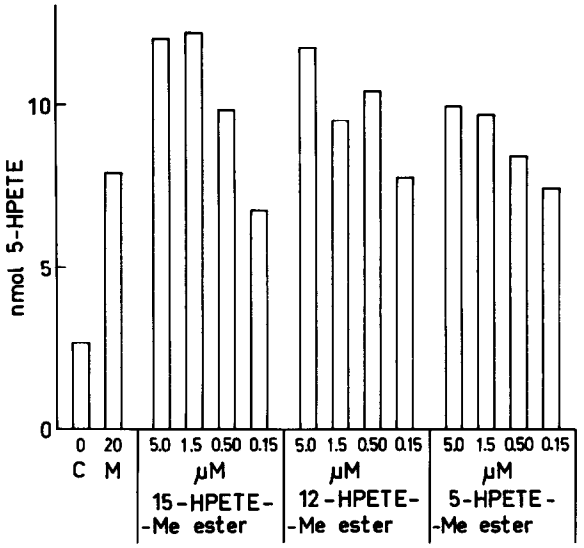

Fig.2. Stimulatory capacity of HPETE methyl esters. Conditions were exactly as described in the legend to fig.1, except that the methyl esters of the HPETEs were used.

ly, or somewhat more active than the free fatty acids (fig.2).

As expected, the corresponding HETEs were totally inactive as stimulators of 5-LO, indicating the need for an intact hydroperoxy function (fig.1). Furthermore, cumene hydroperoxide, *t*-butyl hydroperoxide, and hydrogen peroxide were all ineffective in a concentration range of 1–50 μM, demonstrating that the activating molecule must possess a long-chain fatty acyl moiety.

3.3. *Effect of sulfhydryl reagents on the activation of 5-LO by fatty acyl hydroperoxides*

The role of sulfhydryl reagents in the apparent requirement of 5-LO for exogenous HPETEs was further examined. When a 100000 × *g* supernatant of a leukocyte homogenate was incubated with pure 20:4 in the presence of 1 mM DTT, approx. 3 nmol of 5-HPETE/ml of supernatant were produced within 5 min, and no further production occurred for the remainder of a 3 h incubation period. In contrast, addition of 20 μM mixed HPETEs after 10 min resulted in the formation of 29 nmol of 5-HPETE/ml of supernatant within the ensuing 10 min. A parallel sample, incubated in the absence of DTT produced 12 nmol of 5-HPETE/ml of supernatant during the first 5 min of incubation with pure 20:4. No additional 5-HPETE was produced by this sample during 3 h of further incubation regardless of whether or not HPETEs were added. Thus omission of DTT allowed significant 5-LO activity to occur in the absence of exogenous HPETE. However, DTT had an apparent stabilizing effect on the enzyme, in that samples incubated in the presence of DTT and HPETEs exhibited 1.6–2-fold higher 5-LO activity than samples incubated without DTT in the presence or absence of HPETEs.

4. DISCUSSION

The data presented here suggest that the human leukocyte 5-LO requires fatty acyl hydroperoxide as a stimulating or activating factor. Depending upon the assay conditions, this requirement can manifest itself in a variety of unexpected ways. For example, in the presence of DTT, the 5-LO activities of 10000 × *g* supernatants became highly variable, and those of 100000 × *g* supernatants were unexpectedly low. Such results were readily attributed to enzyme instability, or to inactivation

of the enzyme by the sulfhydryl reagent until the effect of adding exogenous HPETE was realized.

The hydroperoxide activation of the soybean lipoxygenase [10,11], and cyclooxygenase [12] has been known for many years, and more recent studies have provided indirect evidence for a similar phenomenon in the case of mammalian lipoxygenases [13–15]. However, this aspect of the lipoxygenase reaction has been overlooked in most previous studies of the mammalian 5-LO. One possible explanation for this is that many of these studies utilized assays based on thin layer chromatographic or HPLC analysis of products synthesized during incubation of enzyme with radiolabeled substrate. Although these methods provide the high sensitivity and specificity often required for the detection of mammalian lipoxygenases, they are discontinuous techniques and do not allow detailed kinetic analyses such as have been performed for the soybean enzyme.

Our data indicate that failure to recognize the role of HPETEs in the 5-LO reaction may have led to serious misinterpretation of data in the past. This is especially true since many investigators have utilized 100000 × *g* supernatants of leukocyte homogenates, and GSH has often been added to incubation mixtures to prevent the accumulation of potentially damaging HPETE. We have confirmed the beneficial effect of sulfhydryl reagents for stabilizing 5-LO activity, and we routinely include DTT during enzyme purification and assay. Under these conditions, however, the maximal, reproducible analysis of 5-LO activity absolutely requires the routine addition of exogenous HPETE. This is easily and conveniently done by the inclusion of a mixture of nonenzymatically generated HPETEs in the substrate solution as we have described [3].

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REFERENCES

[1] Rouzer, C.A., Matsumoto, T. and Samuelsson, B. (1986) Proc. Natl. Acad. Sci. USA 83, 857–861.
[2] Samuelsson, B. (1983) Science 220, 568–575.
[3] Rouzer, C.A. and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. USA 82, 6040–6044.
[4] Rouzer, C.A., Shimizu, T. and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. USA 82, 7505–7509.
[5] Boeynaems, J.M., Oates, J.A. and Hubbard, W.C. (1980) Prostaglandins 19, 87–97.
[6] Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329–5335.
[7] Yoshimoto, T., Miyamoto, Y., Ochi, K. and Yamamoto, S. (1982) Biochim. Biophys. Acta 713, 638–646.
[8] Corey, E.J., Albright, J.O., Barton, A.E. and Hashimoto, S. (1980) J. Am. Chem. Soc. 102, 1435–1436.
[9] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[10] Gibian, M.J. and Galaway, R.A. (1976) Biochemistry 15, 4209–4214.
[11] Smith, W.L. and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1038–1047.
[12] Hemler, M.E. and Lands, W.E.M. (1980) J. Biol. Chem. 255, 6253–6261.
[13] Sok, D.-E., Han, C.-Q., Pai, J.-K. and Sih, C.J. (1982) Biochem. Biophys. Res. Commun. 107, 101–108.
[14] Maclouf, J., Fruteau de Laclos, B. and Borgeat, P. (1982) Proc. Natl. Acad. Sci. USA 79, 6042–6046.
[15] Siegel, M.I., McConell, R.T., Abrahams, S.L., Porter, N.A. and Cuatrecasas, P. (1979) Biochem. Biophys. Res. Commun. 89, 1273–1280.