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A TEST FOR THE INTERMEDIACY OF 11-HYDROPEROXYEICOSA-5,8,12,14-TETRAENOIC ACID [11-HPETE] IN PROSTAGLANDIN BIOSYNTHESIS

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SUMMARY 11-Hydroperoxy-eicosa-5,8,12,14-tetraenoic acid [11-HPETE] was prepared by chromatographic separation of the hydroperoxides formed from the singlet oxygen oxidation of arachidonic acid [20:4]. 1-[^{14}C]-HPETE was incubated with prostaglandin endoperoxide synthetase preparations from ram seminal vesicles. No prostaglandins products deriving from 11-HPETE were detected in any of the incubations. 11-Hydroxy-eicosa-5,8,12,14-tetraenoic acid [11-HETE], formed by the action of the hydroperoxidase component of prostaglandin endoperoxidase synthetase was the major product formed. The peroxidase activity was absolutely dependent on epinephrine and was stimulated by hematin. 11-HPETE does not appreciably effect the extent of conversion of arachidonic acid into prostaglandin.

INTRODUCTION

Arachidonic acid (20:4)[1] is a substrate for two distinct types of oxygenases in animal tissue. Lipoxygenases in platelets and polymorphonuclear leukocytes catalyze the introduction of one atom of oxygen into 20:4 to form 12-HPETE and 5-HPETE, respectively (1,2). The recent suggestion that 5-HPETE is an intermediate in the formation of the slow reacting substance of anaphylaxis has stimulated interest in animal lipoxygenases and their lipid hydroperoxide products (3). The cyclooxygenase component of prostaglandin endoperoxide synthetase (PES) catalyzes the incorporation of two molecules of oxygen into

1. Abbreviations used: 20:4, arachidonic acid; 12-HPETE, 12-hydroperoxy-eicosa-5,8,10,14-tetraenoic acid; 5-HPETE, 5-hydroperoxy-eicosa-6,8,11,14-tetraenoic acid; PGG_2 15-hydroperoxy-9,11-peroxido-prosta-5-cis, 13-trans-dienoic acid; 11-HPETE, 11-hydroperoxy-eicosa-5,8,12,14-tetraenoic acid; PES, prostaglandin endoperoxide synthetase; PGE_2, 11,15-dihydroxy-9-oxo-prosta-5-cis, 13-trans-dienoic acid; PGD_2, 9,15-dihydroxy-11-oxo-prosta-5-cis, 13-trans-dienoic acid; PGH_2, 15-hydryxy-9,11-peroxido-prosta-5,13-dienoic acid; HHT, 12-hydroxy-heptadeca-5-cis, 8-trans, 10-trans-trienoic acid; $PGF_{2\alpha}$, 9,11,15-trihydroxy-prosta-5-cis, 13-trans-dienoic acid; 11-HETE, 11-hydroxy-eicosa-5,8,10,14-tetraenoic acid.

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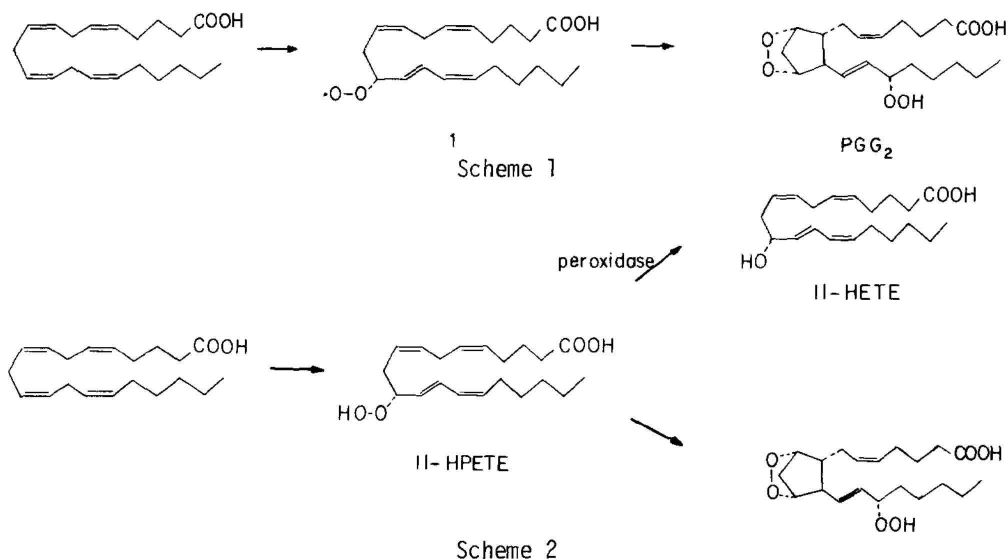
20:4 to form PGG_2, the hydroperoxy endoperoxide intermediate in the biosynthesis of prostaglandins, thromboxanes, and prostacyclin (4).

The mechanism of PGG_2 biosynthesis has been investigated in some detail. It has been suggested that oxygenation of 20:4 at C-11 leads to the peroxy radical intermediate, 1, which then undergoes cyclization to form PGG_2, and model studies have verified the feasibility of Scheme 1 (5,6). Alternatively,



Scheme 1

Scheme 2

one can speculate that 11-HPETE is formed directly by a lipoxygenase type oxygenation and is then converted to PGG_2. This speculation has frequently surfaced but the crucial intermediate, 11-HPETE, has not, until now, been available to test this mechanistic point. We recently prepared 11-HPETE by singlet oxygen oxidation of 20:4 (7) and we report here the results of an investigation designed to determine whether 11-HPETE is an intermediate in PGG_2 biosynthesis.

METHODS

Preparation of 1-[^{14}C]-11-HPETE

A solution of 3.0 mg arachidonic acid [20 Ci] and 1.9 mg methylene blue in 2.5 ml methanol was photolyzed at 0°C for 2 hr and the methanol then removed *in vacuo*. The residue was taken up in 3/1 hexane/ether and chromatographed on 1 gm silica (hexane/ether, 3/1/ vol/vol). The hydroperoxide products were purified by high pressure liquid chromatography (LC) on a Waters Assoc. 10 μ porasil column (9 mm x 30 cm) with acetic acid: isopropanol/hexane, (1/4/995,

vol/vol) at a flow-rate of 5 ml/min. Under those conditions, the retention times of various HPETE isomers were (isomer position; retention time (min.)): 12; 37.3: 14, 15; 40.3: 11; 57.3: 9; 90: 8; 111.

Preparation of PES[1]

Two enzyme preparations were employed in this study. A microsomal fraction from ram seminal vesicles was prepared as previously described (8). A highly purified PES was prepared from the microsomal fraction by detergent solubilization (9), DEAE-cellulose chromatography (10), and isoelectric focusing (11) using the literature methods cited. The pooled active fractions from the isoelectric focusing column were concentrated and the ampholines and diethyldithiocarbamate removed by ultrafiltration. Protein was determined according to Lowry, et al. (12).

Cyclooxygenase Incubations

Incubations were carried out in 0.1 M sodium phosphate (pH 7.8). The enzyme and added cofactors (hematin and epinephrine) were allowed to stand at 25° for 2 min. prior to the addition of lipid. After addition of the lipid, the mixture stood for 3 min at 25° and the reaction was quenched by adding 5 volumes of 50:50 methanol; chloroform followed by 1 volume of 1% aqueous formic acid and 5 volumes of chloroform. The chloroform layer was separated and the volume reduced to 100-200 μl for application to the thin layer plates. Radioactive products were analyzed by autoradiography or by the use of a Thin Layer Radioscanner.

Thin Layer Chromatography: Silica gel 60F-254, 5 cm x 20 cm x 0.25 cm precoated plates were used (Merck & Co., St. Louis, Mo.). The solvent used for elution of the prostaglandins and arachidonic acid alcohols and hydroperoxides was chloroform/methanol/acetic acid/water (90/8/1.0/0.8;, vol/vol). Plates were eluted 15 cm in equilibrated tanks.

RESULTS AND DISCUSSION

1-[^{14}C]-20:4 was reacted with singlet molecular oxygen and the hydroperoxide products were separated by LC as reported. 11-HPETE, prepared by this method had previously been shown to have the 12-*trans*, 14-*cis*,diene stereochemistry (1) as do lipoxygenase products. It should be noted, however, that hydroperoxides prepared by singlet oxygen oxidation are presumably enantiomeric mixtures in contrast to the enantiomerically pure products formed by lipoxygenase.

1-[^{14}C]-11-HPETE was incubated with a microsomal enzyme preparation from ram seminal vesicles and the products compared to those formed from 1-[^{14}C]-20:4. No prostaglandin products were observed using 1-[^{14}C]-11-HPETE as substrate for preparations which converted to 1-[^{14}C]-20:4 predominantly to PGG_2-derived products. Since the localization of PES in microsomal membrane vesicles is unknown, it is possible that it is less accessible to 11-HPETE than to 20:4. We, therefore, performed similar incubations using PES which had been solubilized and purified

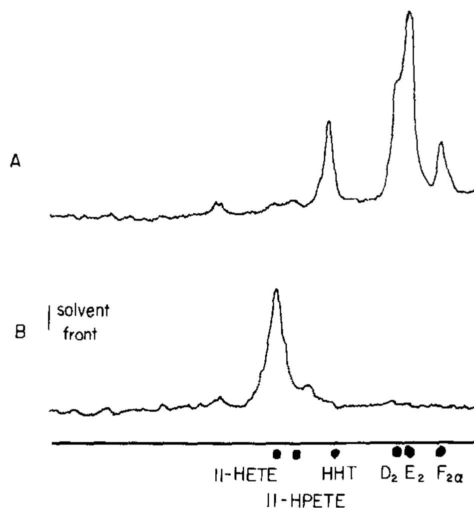


Fig. 1. Thin layer radioscan of PES incubations. A, incubation of 46 μM AA, 1 μM hematin, 500 μM epinephrine, and PES (20 μg/ml). B, incubation of 46 μM 11-HPETE, 1 μM hematin, 500 μM epinephrine, and PES (20 μg/ml).

using literature procedures. Fig 1A displays a thin layer radioscan of the product mixture obtained following incubation of 46 μM 20:4, 1 μM hematin, 500 μM epinephrine, and PES (20 μg/ml). As expected, the primary products are HHT, PGE_2, PGD_2, and $PGF_{2\alpha}$. When 46 μM 1-[^{14}C]-11-HPETE was incubated under identical conditions the radioscan in Fig 1B was obtained. The primary product produced under these conditions was the alcohol, 11-HETE, formed presumably by the hydroperoxidase activity of the purified PES. 11-HETE produced by PES was identical in all respects to material obtained from triphenylphosphine reduction of 11-HPETE. The concentration of 11-HPETE, enzyme, hematin, and epinephrine were independently varied but no PGG_2-derived products were observed from 11-HPETE under any of the conditions chosen.

The cofactor requirements of the hydroperoxidase activity were explored and the results of this study are presented in Fig 2. Epinephrine alone (500 μM) does not appreciably affect the hydroperoxide (Fig. 2A) whereas epinephrine (500 μM) + enzyme (20 μg/ml) (Fig. 2B) leads to a partial, but clean, reduction of 11-HPETE to 11-HETE. Hematin alone (1 μM, data not shown) or hematin (1.0 μM) + enzyme (10 μg/ml) (Fig. 2C) leads to a complex product mixture that contains

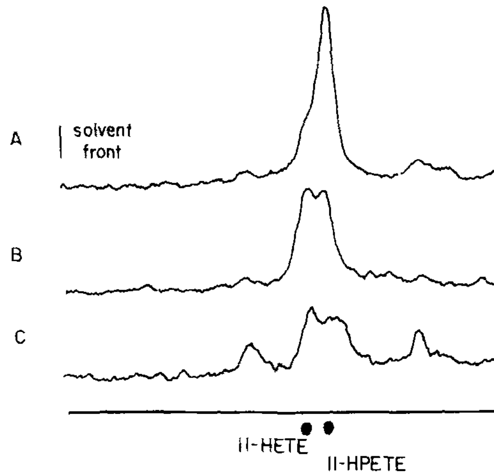


Fig. 2. Co-factor requirements of 11-HPETE and peroxidase. A, 11-HPETE + epinephrine. B, 11-HPETE + Epinephrine + PES. C, 11-HPETE + hematin + PES.

significant amounts of 11-HETE along with other non-prostaglandin products. Boiled enzyme + hematin leads to this same product mixture and this non-enzymatic hematin catalyzed decomposition of 11-HPETE (and the other 20:4 hydroperoxides such as 5-HPETE) will be reported on in detail in subsequent publications. We observe, as others have suggested, that the full activity of the hydroperoxidase requires enzyme + epinephrine + hematin (Fig. 1B) for 11-HPETE reduction.

1-[^{14}C]-20:4 [46 μM], epinephrine [500 μM], hematin [1 μM], PES (20 μg/ml), and unlabelled 11-HPETE [110 M] were reacted and it was noted that the conversion of 20:4 to prostaglandins is clearly not inhibited by the presence of 11-HPETE. In fact, prostaglandin formation from 20:4 is not appreciably affected by 11-HPETE with hydroperoxide concentrations ranging from 4 to 150 μM. The fact that 11-HPETE does not inhibit PGG_2 biosynthesis from 20:4 is important in that it shows that the enantiomer of 11-HPETE with the wrong stereochemistry at C-11 does not inhibit the cyclooxygenase activity. Thus, the fact that 11-HPETE is not a substrate for PES does not relate to the fact that the synthetic 11-HPETE used in the study was a racemic mixture.

The reports that lipoxygenase products (lipid hydroperoxides) account for a significant proportion of 20:4 oxygenation products in platelets and polymorphonuclear leukocytes has renewed speculation that PGG_2 biosynthesis

is initiated by a lipoxygenase type oxygenation at carbon-11. 11-HETE, has, in fact, been isolated in low yield following the incubation of 20:4 with ram seminal vesicle microsomes (13). In addition, 11-hydroxy-eicosadienoic acid is the sole product formed following the incubation of 11,14-eicosadienoic acid (20:2) with homogeneous PES. It has been concluded from a kinetic comparison of the reactions of 20:4 and 20:2 with the purified enzyme that carbon-11 oxygenation is an integral rate-limiting step of PES catalysis rather than a separate reaction resembling that of plant lipoxygenases (14). Nevertheless, the putative intermediate, 11-HPETE has not been available to test this mechanistic point. The results of the present study, in which chemically synthesized 11-HPETE was incubated with microsomal and highly purified PES, clearly demonstrate, 1) that 11-HPETE is not a substrate for PES, and 2) that racemic 11-HPETE does not inhibit the oxygenation of 20:4 by PES. These observations suggest that the biosynthetic proposal presented in Scheme 1 (peroxy radical pathway) is preferred to that presented in Scheme 2 (lipoxygenase pathway). The formation of 11-oxygenated products (11-HETE) during PES catalyzed reactions most likely results from escape of peroxy radical, 1, from the enzyme apparatus followed by hydrogen atom abstraction to form the 11-hydroperoxide. Our observations reported here show that once this hydroperoxide is formed, it cannot be reincorporated into the PGG_2 biosynthetic pathway.

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