Epoxygenation of arachidonic acid by rat anterior pituitary microsomal fractions

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Microsomal fractions isolated from rat anterior pituitary glands catalyze the oxygenation of arachidonic acid. By a combination of chromatographic and mass spectrometric techniques, we have identified epoxyeicosatrienoic acids as reaction products and thus documented the presence of an NADPH-dependent arachidonic acid epoxygenase activity in rat adenohypophysis.

Arachidonic acid *Epoxygenase* *Anterior pituitary* *Epoxyeicosatrienoic acid*

1. INTRODUCTION

We and others have provided in vitro evidence for a new pathway of arachidonic acid metabolism catalyzed by the microsomal monooxygenase system of liver, kidney, brain and lung [1–6]. The reaction has an absolute requirement for cytochrome P-450 [7,8] and utilizes NADPH and oxygen in a 1:1 stoichiometric ratio [1]. In addition to catalyzing the formation of several monohydroxyacids [2,6,9,10], this enzyme system functions as an active 'arachidonic acid epoxygenase' for the formation of 4 novel *cis*-epoxyeicosatrienoic acids (EETs), i.e., 5,6-, 8,9-, 11,12- and 14,15-EET [11]. The EETs are potent mediators for the release of peptide hormones such as somatostatin [5], insulin and glucagon [12], and the anterior pituitary hormone, luteinizing hormone (LH) [13]. More recently, we have documented both the absolute configurations of the EETs [8] and their presence in vivo in rat liver [14].

As a continuation of our studies on the role of arachidonic acid in the mechanism of hormone release from the rat anterior pituitary gland, we report an NADPH-dependent epoxygenase activity in microsomal fractions isolated from rat adenohypophysis.

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2. MATERIALS AND METHODS

Adult Sprague-Dawley male rats (Holtzman, Madison, WI) were killed by decapitation. Anterior pituitaries were dissected from the intermediate and posterior lobes, minced, suspended in cold 0.25 M sucrose and gently homogenized in a glass-teflon homogenizer. Microsomal fractions were isolated by differential centrifugation [15] and suspended in buffer containing 0.15 M KCl, 0.01 M $MgCl_2$, 0.05 M Tris-Cl (pH 7.5), 8 mM sodium isocitrate and 0.25 IU per ml of isocitrate dehydrogenase to a final concentration of 1–2 mg microsomal protein/ml. The microsomal suspensions were incubated with 0.1 mM [1-^{14}C]arachidonic acid (2–4 mCi/mmol) and NADPH (0.5 mM final concentration) for variable time periods at 25°C with constant mixing. After acidification (pH 3), the reaction products were extracted into ethyl acetate and resolved by reverse phase HPLC utilizing solvent mixtures of acetonitrile-water-acetic acid with liquid scintillation monitoring [1]. Metabolites with retention times similar to synthetic *vic*-dihydroxyeicosatrienoic acids (DHETs), hydroxyeicosatetraenoic acids (HETEs), and EETs

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(fractions 60–80, 95–120 and 125–140 respectively, fig.1) were collected batchwise, dried under argon, dissolved in hexane and further resolved by normal phase HPLC on a μPorasil column. The isomeric EETs and HETEs were resolved as in [11] and [9], respectively. DHETs were resolved utilizing a linear gradient of 2.5% isopropanol, 0.1% acetic aid and 97.4% hexane to 5% isopropanol, 0.1% acetic acid and 94.9% hexane over 30 min at a flow rate of 3 ml/min. An aliquot of the crude EET mixture obtained after reverse phase HPLC was methylated as in [11] and analyzed by gas chromatography/mass spectroscopy (GC-MS). Samples were injected onto a 1 m × 2 mm column of 3% SP2100 DOH (Supelco Inc., Bellefonte, PA). The injector, source and transfer line temperature were 235, 250 and 235°C, respectively. Temperature programming was raised from 170 to 210°C at 3°C/min. The carrier gas was CH_4 at a flow rate of 20 ml/min. The eluent was monitored utilizing a Finnigan 4021 mass spectrometer (Finnigan Corp., Sunnyvale, CA) equipped with a Finnigan INCOS 2000 Data System. Positive ion chemical ionization (PCI) spectra were obtained with CH_4.

3. RESULTS AND DISCUSSION

Incubation of [1-^{14}C]arachidonic acid with microsomal fractions, isolated and pooled from 25–30 rat anterior pituitaries, in the presence of NADPH results in the time-dependent formation of a variety of products (fig.1). The appearance of variable amounts of metabolites eluting in fractions 13–40 with retention times similar to prostaglandins (PGs) and in fractions 100–120 does not require NADPH (fig.1). Contamination of the adenohypophyseal microsomal fractions by enzymes from a small population of non-endocrine cells cannot be discarded [16]. There is no arachidonate metabolism using heat denatured microsomal fractions (not shown). By chromatographic comparison with authentic standards, we initially assigned the following oxygenated metabolites in fig.1: DHETs, fractions 60–80; HETEs, fractions 95–120; and EETs, fractions 125–140. The material in fractions 87–90 of fig.1 has a retention time similar to authentic 20-hydroxyeicosatetraenoic acid [6]. Fractions 160–170 contain arachidonic acid.

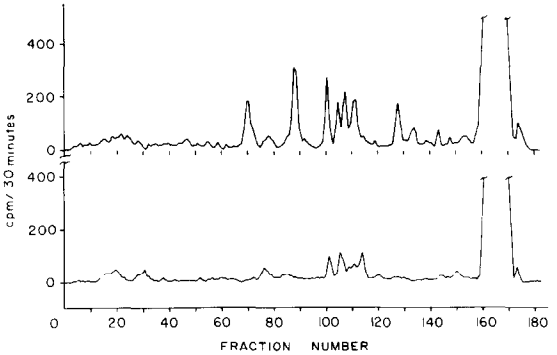

Fig.1. Reverse-phase chromatography of the organic soluble products of arachidonic acid metabolism. Reaction mixtures, each containing microsomal fractions isolated from 25 glands, were incubated at 25°C for 30 min in the presence (top) or absence (bottom) of NADPH. The reaction products were extracted and resolved by HPLC on a μBondapak C_{18} column utilizing a linear solvent gradient from 49.9% H_2O, 0.1% CH_3COOH, 50% CH_3CN to 0.1% CH_3COOH, 99.9% CH_3CN. The rate of change was 1.25% per min at a flow rate of 1 ml per min. Samples (0.2 ml) were collected for liquid scintillation.

The formation of 12- and 15-HETE as well as small amounts of PGE_2, PGD_2 and $PGF_{2\alpha}$ by whole rat anterior pituitaries has been reported [16]. Normal phase HPLC analysis (see section 2) of the combined DHET fractions from reverse phase HPLC revealed metabolites with retention times similar to synthetic 11,12- and 14,15-DHET. Normal phase HPLC analysis also resolved the combined HETE fractions into 5-HETE and its δ-lactone in addition to 12- and 15-HETE [9].

We have reported that in vitro the EETs stimulate the release of LH from cultured rat anterior pituitary cells [12]. A critical step in evaluating a possible in vivo role for the EETs in hormone release is the unequivocal demonstration of a microsomal NADPH-dependent epoxygenase activity in the anterior pituitary. This was established as follows: (i) Normal phase HPLC comparison of the retention times of the radioactive material contained in fractions 125–140 (fig.1) with authentic standards [11] demonstrated the presence of 5,6-, 11,12- and 14,15-EET in a 1.7:1:2.3 ratio, respectively. (ii) Under our conditions of PCI/GC-MS analysis, all 4 synthetic EET methyl esters coelute at approx. 7.4 min [14] and

show a similar fragmentation pattern betwen m/z 250 and 400. Ions in common are m/z 363(M + 29), 335(M + 1), 317, (M − H_2O), 303, (M − CH_3O) and 285, (M − H_2O and CH_3O) [11,14]. An aliquot of the reverse phase HPLC purified EET fraction was esterified with CH_2N_2 [14] and analyzed by PCI/GC-MS as described in section 2. The column eluent was monitored by mass fragmentography at m/z 285, 303, 317, 335 and 363. All 5 EET methyl ester derived ions coelute at the expected retention time (fig.2). The relative intensities are shown by the mass fragmentogram constructed with the above data (fig.2).

An estimation of the relative distribution of products produced from arachidonic acid by rat pituitary microsomes in the presence of NADPH is presented in table 1. The data are the mean values from 3 microsomal preparations. Lipoxygenase-like activity is the major arachidonic oxygenation reaction catalyzed by pituitary microsomes (55% of the total) [16]. Epoxygenation as measured by EET and DHET production accounts for approx. 29% of the reaction products. PG-like metabolites

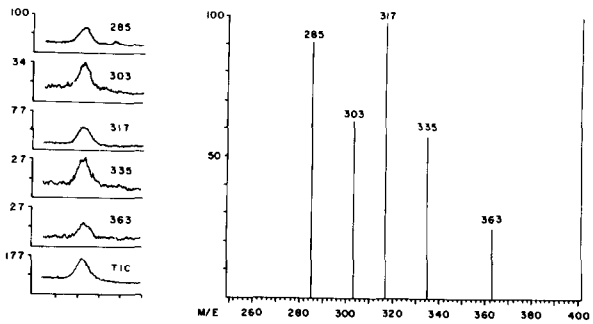

Fig.2. Mass fragmentographic analyses of the EET sample purified from rat anterior pituitary microsomal incubates. The radioactive material in fractions 125–140 of the chromatogram shown in fig.1 was collected and concentrated under a stream of argon. After methylation with CH_2N_2, an aliquot was submitted to GC under the conditions indicated in section 2. The GC column eluent was monitored by PCI/mass fragmentography at m/z 285, 303, 317, 335 and 363. Left panel: GC elution profile of the selected ions as well as the total ion current (TIC). Right panel: mass fragmentogram constructed with the data. Abscissa: mass scale m/z; ordinate: abundance in percent of the base peak.

Table 1

Product distribution from arachidonic acid oxygenation by anterior pituitary microsomes

Type	Compound	% of total[c]
HETE[a]	15-HETE	10
	12-HETE	25
	5-HETE	12
EET/DHET[a]	14,15-DHET	10
	11,12-DHET	4
	5,6-EET	5
	11,12-EET	3
	14,15-EET	7
ω[b]	20-hydroxy	16
Unknown		8

[a] Metabolites tentatively identified by coelution with authentic synthetic standards in normal and reverse phase HPLC

[b] Tentatively identified by coelution with synthetic 20-hydroxyeicosatetraenoic acid in reverse phase HPLC

[c] Mean values from 3 experiments. The standard deviations are within 15–20% of the average

The values correspond to the percent distribution of metabolites eluting from fractions 40 to 140 of the reverse phase HPLC (fig.1)

varied from 1 to 20% of the total products. Excluding PG-like metabolites, microsomal fractions derived from 50 anterior pituitary glands metabolize arachidonic acid at a total rate of approx. 0.2 nmol per min at 25°C.

In summary, this study documents the presence of an arachidonic acid epoxygenase activity in microsomal fractions isolated from rat anterior pituitary glands. These results expand the list of tissues capable of supporting the epoxygenation of arachidonic acid and suggest a possible role for this pathway in the process of anterior pituitary hormone release.

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