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FORMATION AND METABOLISM OF HEPOXILIN A3 BY THE RAT BRAIN

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Incubation of homogenates of the rat cerebral cortex with arachidonic acid led to the appearance of hepoxilin A3, analysed as its stable trihydroxy derivative, trioxilin A3, by high resolution gas chromatography / electron impact mass spectrometry. Using the stable deuterium isotope dilution technique, it is estimated that the cerebral cortex generates 5.0 ± 0.2 ng / mg protein of hepoxilin A3. The formation of this product was stimulated by the addition of exogenous arachidonic acid (12.9 ± 1.5 ng / mg protein) and blocked by boiling of the tissue. Addition of the dual cyclooxygenase / lipoxygenase inhibitor BW 755C at a concentration of 75 uM did not result in a blockade of hepoxilin formation. Three other regions were also tested for their ability to form hepoxilin A3 upon stimulation with exogenous arachidonic acid, i.e. median eminence, 11.7 ± 1.6 ng / mg protein, pituitary, 12.3 ± 0.7 ng / mg protein; pons, 26.6 ± 0.2 ng / mg protein. In a separate study, 14C-labelled hepoxilin A3 was transformed into 14C-labelled trioxilin A3 by homogenates of the rat whole brain, demonstrating the presence of epoxide hydrolases in the CNS which utilise the hepoxilins as substrates. This is the first demonstration of the occurrence of the hepoxilin pathway in the central nervous system.

The hepoxilins constitute a recently described class of lipids derived from arachidonic acid (1,2) and eicosapentaenoic acid (3) containing both hydroxyl and epoxy functional groups. Hepoxilins A3 and B3 are formed from the fatty acid precursor via the 12-lipoxygenase system through a unique intramolecular rearrangement of the 12-hydroperoxy product, 12-HPETE (4). Hepoxilin A3 is rapidly hydrolysed to the trihydroxy product, trioxilin A3, during mild acid treatment or through reaction with a specific epoxide hydrolase (5). Hepoxilin B3 is more stable to acid catalysed and enzymatic hydrolysis (1) and has been isolated intact (1,6-8). The hepoxilins possess insulin secretagogue activity (2,6) and facilitate the transport of Ca^{+2} across membranes (9). The present study was undertaken to investigate whether the hepoxilin pathway (formation and degradation) occurs in brain tissue.

Abbreviations used: GCMS, gas chromatography -mass spectrometry; HxA3, hepoxilin A3, 8-hydroxy-11,12-epoxy-eicosa-5Z, 9E, 14Z-trienoic acid; TrXA3, trioxilin A3, 8,11,12-trihydroxy-eicosa-5Z, 9E, 14Z-trienoic acid; HxB3, hepoxilin B3, 10-hydroxy-11,12-epoxy-eicosa-5Z, 8Z, 14Z-trienoic acid; TrXA3, 10,11,12-trihydroxy-eicosa-5Z,8Z,14Z-trienoic acid; Me, methyl ester; t-BDMSi, tertiary butyl dimethylsilyl ether.

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MATERIALS AND METHODS

Materials: Deuterium (d4)-labelled HxA3 (deuterium atoms at positions 5,6,14,15) was kindly provided as the methyl ester derivative by Professor E. J. Corey. It was saponified to the free acid by reaction with ethanol / 1N sodium hydroxide (1/1, v/v) at room temperature and was subsequently hydrolysed to the stable trihydroxy derivative, TrXA3, by acidification to pH 3 with 1N HCl prior to extraction. 14C-labelled HxA3 was biosynthesised from 14C-labelled arachidonic acid as previously described (1). Arachidonic acid was purchased from Sigma, St. Louis, MO; it was dissolved in ethanol, and added in 5 ul of this vehicle to the incubations. BW 755C was a generous gift of Dr. S. Moncada, Burroughes-Wellcome, Kent, U.K.

Incubations: Adult male Wistar rats (Charles River) weighing 250-300 g were used in these experiments. Rats were sacrificed by cervical dislocation, the brain was dissected and a portion of the cerebral cortex, the median eminence, pituitary and pons was removed. The tissues were thoroughly rinsed with ice-cold 100 mM Tris-HCl buffer (pH 9) and homogenised separately (Polytron, Brinkman) in the same buffer. The homogenate was centrifuged at 2°C at 2000 rpm and an aliqout of the supernatant was taken for protein determination. The enzyme solution was diluted to a concentration of 1 mg protein / ml and 0.5 ml of this solution was incubated in air with rapid shaking at 37°C in the presence and absence of 10 ug arachidonic acid, in the presence and absence of BW 755C (10 ug). For metabolism studies the homogenate was incubated with 14C-labelled HxA3 (5000cpm, approx. 10ng) for 5 minutes at 37°C. The incubation was terminated at 20 minutes by the addition of 5 ml of absolute ethanol and the precipitated protein was removed by centrifugation. The supernatant was transferred, 20 ng of d4-labelled trioxilin A3 was added as internal standard, and after mixing, the solution was diluted with 10 volumes of water and acidified to pH 3 with 1N HCl. The acidified solution was passed through a C18 SEP PAK (Waters / Millipore, Millford, MA) prewashed with methanol and water. After sample loading, the SEP PAK was eluted with 10 ml water, 10 ml methanol / water / acetic acid (10 / 90 / 0.4, v/v) then 5 ml of methanol / water / acetic acid (65 / 35 / 0.4, v/v). The trioxilins were eluted with the latter solvent. This fraction was taken to complete dryness in vacuo and the residue was dissolved in 100 ul methanol for subsequent derivatisation.

Samples in which the metabolism of HxA3 was investigated were acidified to pH 3 with 0.5N HCl and extracted with diethyl ether. The ether extract was taken to complete dryness in vacuo, and the residue was dissolved in a small volume of diethyl ether / methanol (1/1, v/v). The sample was spotted on silica gel thin layer plates (Brinkman) and developed with ethyl acetate / acetic acid (99 / 1, v/v). The radioactive products were visualised by scanning of the plates with a Berthold thin layer chromatoplate scanner. The appropriate zones were scraped into scintillation vials, 1 ml of methanol / water (1/1, v/v) was added to elute the products from the silica gel, and 10 ml of PCS scintillation cocktail was added. Radioactivity was quantified with a Beckman liquid scintillation spectrophotometer.

Derivatisation: The samples, dissolved in 100 ul methanol, were converted into methyl esters by reaction with 900 ul of a freshly prepared solution of diazomethane in diethyl ether. After 15 minutes at 23°C in the dark, the solution was taken to dryness with a stream of nitrogen gas. The residue was dissolved in 100 ul of t-BDMSi reagent (Supelco Inc., Bellefonte, PA) and heated at 70°C for 45 minutes. The sample was subsequently cooled on ice and 2 ml of hexane was added. After gentle mixing the hexane layer was transferred, 1 ml of water was added to it and after thorough mixing, the hexane was transferred and taken to dryness with a stream of nitrogen gas. The residue was taken up in 1 ml of iso-octane and transferred to a small pasteur pipette column half filled with silicic acid (BioSil HA, minus 320 mesh, BIORAD, Richmond, CA). The column was eluted with 10 ml of iso-octane to remove contaminating material and the trioxilins were subsequently eluted with 4 ml of benzene. This solution was taken to dryness with a stream of nitrogen gas and the residue was dissolved in 10 ul of diethyl ether for immediate analysis by GCMS.

GCMS: A Hewlett-Packard 5970 benchtop GCMS (MSD) was used, equipped with a 60 meter capillary column (DB-1, J & W Scientific, Rancho Cordova, CA) connected into the source. Hydrogen was employed as carrier gas at a linear velocity of 30 cm / sec at 50°C. The mass spectrometer was operated in the electron impact mode (70 eV) using selected ion monitoring at an electron multiplier voltage of 2800. Source pressure was maintained at 8.10^{-5} Torr. The sample was injected in 2 ul of diethyl ether directly on column at an oven temperature of 50°C. After 4 minutes the oven temperature was raised at 20°C / minute to a final temperature of 300°C where it was maintained until the end of the run. Under these conditions the trioxilins were eluted as a quartet with a retention time between 30 - 34

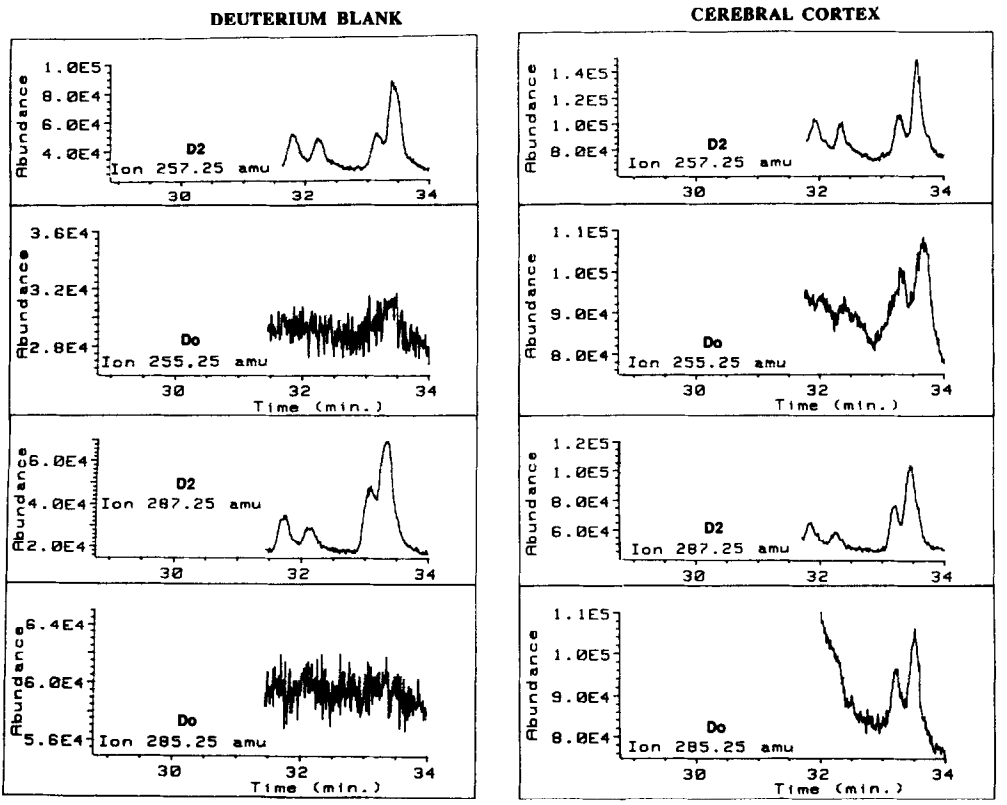

Figure 1. High resolution GC-electron impact MS selected ion chromatograms showing profiles of deuterium labelled internal standard (m/z 257, 287) of trioxilin A3 and undeuterated (m/z 255, 285) product present in: Left panels, internal standard (deuterium labelled) alone and Right panels, internal standard mixed with extract of brain cerebral cortex incubated with 10 ug arachidonic acid. Products were assayed as the Me t-BDMSi derivatives.

minutes. Sample quantitation was achieved by selected ion monitoring, using two prominent ion fragments in the trioxilin mass spectrum i.e. m/z 257 and 287 for the deuterated (d2) species (internal standard), and 255 and 285 for the endogenous (d0) species. Quantitation was achieved by measurement of the peak height of the d0 species divided by the peak height of the d2 species, multiplication of the product by 20, the amount of the deuterated internal standard added, and division by the amount (mg) of tissue protein used. Results are expressed in ng / mg protein. The mean of duplicate assays is repoorted.

RESULTS AND DISCUSSION

The electron impact mass spectrum of trioxilin A3 as the Me t-BDMSi derivative is dominated by two major fragment ions, i.e. m/z 285 due to the fragment consisting of C1 to C8, and m/z 255 due to the C12-C20 fragment of the molecule (10). The corresponding fragments resulting from the deuterium-labelled internal standard occur at m/z 287 and 257 respectively (unpublished). Using deuterium labelled trioxilin A3 as internal standard we investigated the formation of hepoxilin A3 by the rat brain with high resolution GC combined with MS operated in the electron impact mode. Further identification of trioxilin A3 by mass spectrometry is confirmed by the quartet of peaks observed for this product on capillary GC at defined retention times. As shown in Figure 1 (left panels), authentic d4 TrXA3 displays

Table 1. Formation of hepoxilin A3 (measured as trioxilin A3) by rat brain homogenates

Brain Region	Condition	TrXA3 formed assayed by GCMS (ng / mg protein)
Cerebral Cortex	Control*	5.0 ± 0.2
	Control + Arachidonic acid (10ug)	12.9 ± 1.5
	Control + Arachidonic acid (10ug) + BW 755C (10 ug)	16.1 ± 0.3
Median Eminence	Control + Arachidonic acid (10ug)	11.7 ± 1.6
Pituitary	Control + Arachidonic acid (10ug)	12.3 ± 0.7
Pons	Control + Arachidonic acid (10ug)	26.6 ± 0.2

*Control incubations refer to incubations of 0.5 ml of supernatant (2000 rpm) (500ug protein) in 100 mM Tris-HCl buffer, pH 9 in the absence of any additions. All incubations were carried out for 20 minutes in air at 37°C, and the product was quantitated by EI-GCMS using the SIM technique. Values represent the mean of duplicates.

four major peaks at retention times 31.8, 32.4, 33.3 and 33.6 minutes. At the amount of material injected on column (2.5 ng), there is virtually no detectable undeuterated blank as monitored by the mass peaks at m/z 285 and 255. The corresponding profiles of the incubated cerebral cortex to which was added 20 ng of deuterated internal standard (Fig 1, right panels) show the deuterated sample (m/z 287 and 255) and the corresponding unlabelled products formed from the added arachidonic acid (m/z 285 and 255) by the brain enzymes. Quantitation of the product formed reveals an amount of trioxilin A3 (and hence hepoxilin A3) of 5.0 ± 0.2 ng / mg protein / 20 min at 37°C in the unstimulated condition.

The properties of the cerebral cortex enzymes to form hepoxilins were further investigated after addition of exogenous arachidonic acid, in the presence and absence of the dual cyclooxygenase and lipoxygenase blocker (BW 755C)(11-14) and after tissue boiling. As shown in Table 1, addition of exogenous arachidonic acid greatly stimulated the formation of hepoxilin A3, while BW 755C, at the concentration used, did not affect the arachidonic acid-stimulated formation of hepoxilin A3 suggesting that unlike reports with platelet 12-lipoxygenase (11-14), the brain enzyme is not sensitive to blockade by BW-755C.. Boiling of the tissue prior to incubation resulted in amounts of hepoxilin A3 detected similar to the unstimulated controls (data not shown). These data demonstrate that hepoxilin A3 (detected as trioxilin A3) is formed by the cerebral cortex. Three other regions were tested for their ability to form hepoxilin A3 in the presence of exogenous arachidonic. Of these the highest activity was found in the pons (Table 1).

The capacity of rat brain to metabolise the hepoxilins was evaluated by incubating 14C-labelled HxA3 with homogenates of the whole brain. Figure 2 shows thin layer chromatograms of extracts

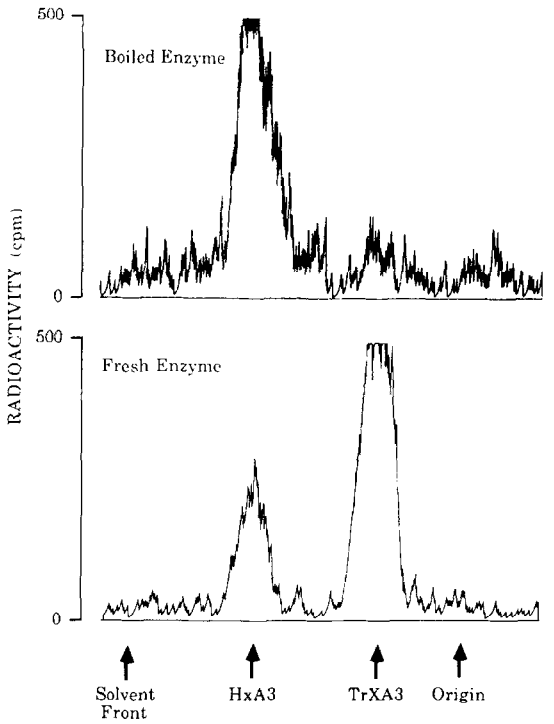

Figure 2. Thin layer radiochromatograms showing the enzymatic conversion of 14C-labelled HxA3 into TrXA3 by homogenates of rat whole brain.

derived from incubation of substrate with a) boiled enzyme and b) fresh enzyme. It is clear from these chromatograms that HxA3 is converted into TrXA3 only with the native enzyme demonstrating a heat-sensitive epoxide hydrolase as we have previously demonstrated in the rat lung (1) and blood platelets (5). The metabolism of HxA3 is dependant on the protein concentration (data not shown).

This is the first demonstration of the presence of the hepoxilin pathway (formation and degradation) in the CNS. While hepoxilins have recently been shown to release insulin from isolated perifused pancreatic islet cells and to facilitate the transport of calcium across membranes (2,9), their effects in the CNS still remain to be determined. A recent report has shown that sensory neurons from the Aplysia possess 12-lipoxygenase activity and that 12-HPETE, but not 12-HETE, is capable of mediating inhibitory presynaptic responses to the tetrapeptide, FMRFamide, in these neurons. Whether this action of 12-HPETE is exerted on its own or after its conversion into the hepoxilins (Figure 3) must await further studies.

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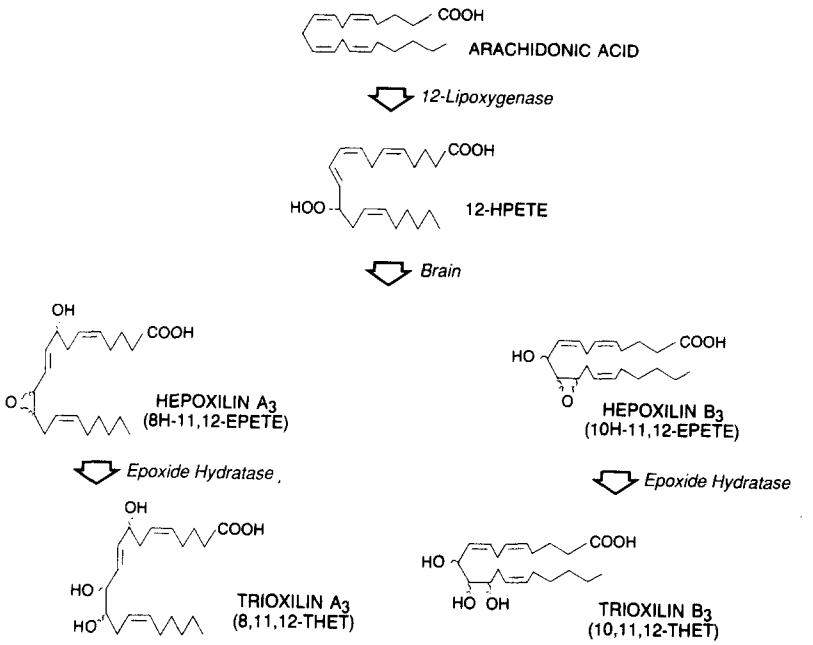

Figure 3. Scheme showing the biosynthetic pathway in the formation of the hepoxilins and trioxilins in the rat brain. Hepoxilin A3 formed was analysed as the corresponding trioxilin A3. Hepoxilin B3 and trioxilin B3 were not investigated in this study.

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