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Brain uptake and metabolism of the endocannabinoid anandamide labeled in either the arachidonoyl or ethanolamine moiety☆

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ABSTRACT

Introduction: Anandamide (N-arachidonoylethanolamine) is a retrograde neuromodulator that activates cannabinoid receptors. The concentration of anandamide in the brain is controlled by fatty acid amide hydrolase (FAAH), which has been the focus of recent drug discovery efforts. Previous studies in C57BL/6 mice using [^{3}H-arachidonoyl]anandamide demonstrated deposition of tritium in thalamus and cortical areas that was blocked by treatment with an FAAH inhibitor and that was not seen in FAAH-knockout mice. This suggested that long chain fatty acid amides radiolabeled in the fatty acid moiety might be useful as *ex vivo* and *in vivo* radiotracers for FAAH, since labeled fatty acid released by hydrolysis would be rapidly incorporated into phospholipids with long metabolic turnover periods.

Methods: Radiotracers were administered intravenously to conscious Swiss–Webster mice, and radioactivity concentrations in brain areas was quantified and radiolabeled metabolites determined by radiochromatography.

Results: [^{14}C]Arachidonic acid, [^{14}C-arachidonoyl]anandamide and [^{14}C-ethanolamine]anandamide, and also [^{14}C]myristic acid, [^{14}C-myristoyl]myristoylethanolamine and [^{14}C-ethanolamine]myristoyl-ethanolamine all had very similar distribution patterns, with whole brain radioactivity concentrations of 2–4% injected dose per gram. Pretreatment with the potent selective FAAH inhibitor URB597 did not significantly alter distribution patterns although radiochromatography demonstrated that the rate of incorporation of label from [^{14}C]anandamide into phospholipids was decreased. Pretreatment with the muscarinic agonist arecoline which increases cerebral perfusion increased brain uptake of radiolabel from [^{14}C]arachidonic acid and [^{14}C-ethanolamine]anandamide, and (in dual isotope studies) from the unrelated tracer [^{125}I]RTI-55.

Conclusions: Together with our previously published study with [^{18}F-palmitoyl]16-fluoro-palmitoylethanolamine, the data show that the primary determinant of brain uptake for these tracers in Swiss–Webster mice is initial distribution according to blood flow. It is possible that recently reported strain differences in long chain fatty acid trafficking between C57BL/6 and Swiss–Webster mice are responsible for the differences between our results using [^{14}C]anandamide and the earlier studies using [^{3}H]anandamide.

1. Introduction

Anandamide (*N*-arachidonoylethanolamine) is a lipid signaling molecule that is an agonist at cannabinoid and vanilloid receptors [1]. In the brain endocannabinoid system it acts as a retrograde neuromodulator, being produced post-synaptically and activating presynaptic cannabinoid CB1 receptors that control release of classical neurotransmitters [2]. Fatty acid amide hydrolase (FAAH) is primarily responsible for terminating actions of anandamide and other fatty acid ethanolamides) in the brain [3]. Potent and selective inhibitors of this serine hydrolase have been developed, including [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597) [4], and the enzyme has been the subject of much drug discovery effort [5,6]. It is possible that an inhibitor of FAAH, by raising cannabinergic tone only in brain regions that are producing anandamide, might provide some of the therapeutic actions ascribed to delta-9 tetrahydrocannabinol (the principal active component of cannabis) without its undesirable psychoactive effects. Interest in the therapeutic potential of FAAH inhibitors for indications such as pain and psychiatric disorders has led to the development of radiolabeled analogs of these compounds that can be used to examine the distribution of FAAH in brains of living humans and other animals, so that the relationship between dose regimens and *in vivo* binding of candidate drugs to FAAH activity can be imaged [7,8]. A potential alternative approach to the use of radiolabeled irreversibly binding FAAH inhibitors would be the use of labeled substrates of FAAH that yielded metabolically trapped labeled products. These might give complementary information, related to flux through FAAH rather than the

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concentration of FAAH, to that obtained using the radioligand approach. Glaser and co-workers provided evidence that this approach was feasible [9]. They administered anandamide labeled with tritium on the arachidonic acid moiety to C57BL/6 mice, and found that the brain concentration of tritium was about 2.5% ID/g at euthanasia times between 5 and 60 min; this was 3–4 times higher than seen when [^{3}H]arachidonic acid was administered. Autoradiograms of sections prepared from brains of mice euthanized at 30 min, obtained using a Biospace beta-imager, revealed a pattern of tritium deposition for [^{3}H]anandamide that was distinct from that obtained with tritiated arachidonic acid, with highest radioactivity levels (30–40% higher than in striatum or hippocampus) in somatosensory and visual cortex and thalamus. Furthermore, in FAAH knockout mice, selective uptake of radiolabel in cortex and thalamus was eliminated and at 30 min post injection tritium in the brain remained in the form of anandamide. Addition of the serine hydrolase inhibitor methyl arachidonoylfluorophospate (MAFP) to the intravenous injection mixture also reduced radiolabel in the brain and increased the fraction that was unmetabolized anandamide [9]. The trapping mechanism in these experiments is formation of glycerophospholipids from labeled arachidonic acid released from anandamide by the action of FAAH. Arachidonic acid is rapidly esterified at the 2-position of phospholipids, and is released from this pool quite slowly; the turnover time is 3–4 h [10]. The brain regional disposition of label from intravenously administered arachidonic acid in the rat brain has been evaluated in a series of publications from the Rapoport group [11–13].

We recently evaluated [^{18}F]16-fluorohexadecanoylethanolamine ([^{18}F]omega-fluoropalmitic acid; FHEA) as a positron labeled analog of labeled anandamide [14]. Palmitoylethanolamine is also a substrate for FAAH [15], is present in the brain in higher concentrations than anandamide [16], and is marketed as a nutraceutical [17]. Measuring ^{18}F in microdissected brain regions of Swiss–Webster after intravenous injection of FHEA, we found that: (1) brain concentration of ^{18}F was 3–4 %ID/g at all times evaluated (1, 5, 15, 30, 60 min), with no time-dependency; (2) ^{18}F concentrations in hippocampus were lower than those in striatum, cerebellum thalamus, cortex or brain stem at all times tested; (3) there were no significant differences in brain regional content of ^{18}F measured at 30 min between mice pretreated with the potent selective FAAH inhibitor URB597 and vehicle-pretreated mice; (4) radio thin-layer chromatography of brain tissue 30 min after euthanasia showed significantly higher intact FHEA and significantly lower phosphatidylcholine levels in URB597-pretreated animals compared with vehicle-treated animals. Thus while URB597 did inhibit FAAH *in vivo*, it did not reduce regional brain concentrations of radiolabel from a radioactive substrate of FAAH whose metabolic product was incorporated in phospholipids. These results with [^{18}F]fluoropalmitoylethanolamine [14] appear to stand in contrast to those of Glaser et al. [9] with [^{3}H-arachidonoyl]ethanolamine and are disappointing from the standpoint of developing a metabolically trapped PET tracer for FAAH.

In the present study, we evaluated the effects of URB597 on *in vivo* metabolism and brain regional deposition of [^{14}C]anandamide, with the radiolabel being either on the arachidonic acid moiety or the ethanolamine moiety. Similar studies were conducted with [^{14}C]myristoylethanolamine. We also evaluated the effects of the cholinergic muscarinic agonist arecoline on brain radioactivity concentrations at early times after administration of [^{14}C]arachidonic acid or [^{14}C]anandamide. Arecoline increases brain perfusion and also is reported to stimulate phospholipase A_2, the enzyme that mobilizes arachidonic acid from phospholipids [18–20]. In these experiments we included the labeled cocaine analog [^{125}I]RTI-55 (2-beta-carbomethoxy-3-beta-[4-iodophenyl]tropane) [21] in the injected material. While RTI-55 labeled with ^{125}I or ^{123}I is generally employed as a radiotracer for monoamine reuptake sites, it is well-extracted by the brain [22] and was used here at early experimental time-points to indicate changes is cerebral perfusion.

2. Materials and methods

2.1. Animals

Male Swiss–Webster mice (25g) were purchased from Taconic Farms. They were maintained at the animal facility of Division of Laboratory Animal Medicine (DLAM) on 12 h alternating light and dark periods, with access to food and water *ad libitum*. Mice were treated humanely in compliance with NIH guidelines for the use of laboratory animals, and according to a protocol approved by Northeastern University Institutional Animal Care and Use Committee (IACUC). They were housed in groups of 5 in a facility until used. Radiotracers were injected intravenously in a 0.2 mL volume of vehicle consisting of ethanol/emulphor/0.9% saline (1:1:18, v/v) after placement in a mouse holder (Braintree Scientific). At indicated times they were euthanized by cervical dislocation, following which the brain was rapidly removed.

2.2. Assay of brain-regional radioactivity after microdissection

In some experiments, the brain was dissected into major regions using the forceps method. Iodine-125 was assayed using a gamma-counter (COBRA). For assay of carbon-14 or tritium, brain regions were dissolved using "Solvable" (Perkin-Elmer) before addition of UltimaGold liquid scintillation fluid; samples were then counted using a Beckman 6500 instrument. Samples of whole blood were decolorized by addition of 50 microliters of hydrogen peroxide before addition of liquid scintillation fluid.

2.3. Autoradiography

Brains were placed in ice-cold saline, blotted, and after removal of the cerebellum and brain stem they were glued to the stage of a vibratome (World Precision Instruments) so that coronal sections of width 300 could be prepared. Sections were air-dried and then apposed to phosphorimaging plates. Plates were developed using a Cyclone system (Perkin-Elmer), and images were analyzed using the ImageQuant software supplied with the instrument.

2.4. Radiotracers

Carbon-14 and tritium labeled compounds were purchased from American Radiolabeled Chemicals or from Maravek. Fatty acid ethanolamides were prepared from radioactive ethanolamine and appropriate carboxylic acids using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as a carboxyl activating agent and 4-dimethylaminopyridine as a catalyst. Dichloromethane was used as solvent and reactions were done under argon. Compounds were purified using silica gel chromatography.

Iodine-125 iodide was purchased from Perkin-Elmer; [^{125}I]RTI-55 was prepared by iododestannylation [23], using trimethyltin starting material purchased from ABX Advanced Biochemical Compounds. It was purified by reverse-phase HPLC. Radioactive peaks were collected and partitioned between ether and water. The ether layer was dried using anyhydrous sodium sulfate, and the solvent removed under a stream of argon. The residue was redissolved in ethanol and stored at −20 degrees until use.

2.5. Radioanalytical studies

A modified Folch procedure [24] was used to extract radioactive species from brain or blood. Organic fractions were analyzed using silica gel thin-layer chromatography. Aqueous fractions were analyzed using cation exchange HPLC.