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Occurrence and postmortem generation of anandamide and other long-chain *N*-acylethanolamines in mammalian brain

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Abstract Long-chain *N*-acylethanolamines (NAEs) were assayed in pig, sheep and cow brain by gas chromatography/mass spectrometry of their *tert.*-butyldimethylsilyl derivatives in the presence of deuterium-labeled internal standards. Immediately after death, total NAEs ranged from about 2.7 μg/g wet weight (sheep, cow) to 6.5 μg/g wet weight (pig) and consisted almost exclusively (99%) of saturated and monounsaturated species. Anandamide (20:4n-6 NAE) comprised about 1% of total NAE in pig and cow brain, but was absent in freshly extracted sheep brain. When pig brain was analysed after 0.5, 1, 3, 4 and 23 h at ambient temperature, NAE levels were found to increase substantially over the entire time period with 20:4n-6 NAE formation exhibiting a time lag compared to that of saturated and monounsaturated NAEs.

Key words: *N*-Acylethanolamine; Anandamide; Arachidonic acid; Pig brain; Sheep brain; Cow brain

1. Introduction

Devane et al. [1] have isolated *N*-arachidonoylethanolamine (arachidonoylethanolamide, 20:4n-6 NAE) from pig brain and determined that it binds to the brain's cannabinoid receptor. Other long-chain polyunsaturated *N*-acylethanolamines have been identified in pig brain as well [2,3] and some were reported to exhibit similar receptor interaction [4], whereas the saturated analog, *N*-palmitoylethanolamine, was found to be inactive [1,4]. *N*-Arachidonoylethanolamine, termed 'anandamide' [1], was also shown to inhibit both adenylate cyclase [5] and calcium currents [6], and to display other pharmacologic activities [7–9]. As potentially psychoactive agents, long-chain polyunsaturated NAEs would be expected to be readily generated and degraded, perhaps by direct *N*-acylation and deacylation of ethanolamine [10–15], and to be present only in trace amounts in vivo. However, postdecapitative cerebral ischemia is known to cause a rapid rise in free fatty acid levels in rat brain [16–19], including the polyunsaturated fatty acids 20:3n-6, 20:4n-6 and 22:6n-6, all of which have been found as ethanolamides in pig brain extracts [1,2]. This raises questions regarding possible postmortem generation of NAEs in mammalian brain.

We have previously identified long-chain NAEs and their precursors, *N*-acylethanolamine phospholipids, in infarcted dog heart [20,21] and have observed that *N*-acylethanolamine phospholipids are generated in young rat brain within 15 min postmortem [19]. In mammalian cells, *N*-acylethanolamine phospholipids are produced by acyl transfer from the *sn*-1 position of various glycerophospholipids to the free amino group of ethanolamine phospholipids [22–24], a reaction that does not involve ester hydrolysis [25]. They are metabolized to *N*-acylethanolamines and phosphatidic acid by phosphodiesterase (phospholipase D) activity [26,27]. This pathway generates primarily saturated and monounsaturated, as well as some diunsaturated [28], NAEs and these are the only ones for which quantitative information is available [29].

Here, we report the quantitative assay of NAEs present in pig, sheep and cow brain at different periods postmortem using gas chromatography-mass spectrometry of their *tert.*-butyldimethylsilyl derivatives in the presence of deuterated analogs as internal standards.

2. Materials and methods

2.1. Brains

Pigs, sheep and cows were killed by exsanguination and brains were removed immediately. 2–4 min after death the brains were either frozen in dry ice or homogenized in chloroform. In some cases, the intact brains were stored in plastic bags at ambient temperature (22°C) for periods of 0.5–23 h before being frozen or extracted. No attempt was made to separate white from gray matter or cerebrum from cerebellum.

2.2. *N*-Acyl[$^2H_4$]ethanolamine standards

Deuterated NAEs were prepared from 1,1,2,2,-[$^2H_4$]ethanolamine (Isotec, Miamisburg, OH) and the corresponding acyl chlorides (NuChek Prep, Elysian, MN) by a modification of the procedure of Roe et al. [30]. Ethanolamine (1.6 mmol), acyl chloride (0.4 mmol) and pyridine (6 mmol) were mixed in 1 ml of toluene in a small screw-cap tube and heated to 80°C for 1–2 h. After cooling, most of the toluene was removed under a stream of nitrogen and 5 ml of chloroform was added. The chloroform solution was applied to a column of 5 g of silicic acid (Davisil type 634; Aldrich Chemical, Milwaukee, WI) prepared in chloroform. Unreacted fatty acid was eluted with 50 ml of chloroform and the NAE was eluted with 50 ml of 2% methanol in chloroform, collected in 10-ml fractions. Reaction yields (50–65%) and purity of the column fractions were checked by TLC on silica gel H using hexane/diethyl ether/acetone/acetic acid (40:20:30:1, v/v/v/v) as developing solvent. TLC fractions were made visible by charring after spraying the plates with 50% H_2SO_4. *N*-Arachidonoylethanolamine was purchased from Matreya (Pleasant Gap, PA). Other unlabeled NAEs were prepared as described above. All synthetic NAEs were characterized by the GC retention times and mass spectra of their *tert.*-butyldimethylsilyl derivatives (see below).

2.3. Lipid extraction and fractionation

Brain tissue (20 g) was homogenized in 180 ml of chloroform with a Tissumizer (Tekmar, Cincinnati, OH). A mixture of *N*-acyl[$^2H_4$]ethanolamines (100 μg each of 16:0, 18:0, 18:1n-9 and 20:4n-6 NAE) was added during homogenization. The chloroform homoge-

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Abbreviations: NAE, *N*-acylethanolamine; TLC, thin-layer chromatography; GC-MS, gas chromatography-mass spectrometry; MSD, mass selective detector; *t*-BDMS, *tert.*-butyldimethylsilyl.

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nate was centrifuged in a JA-14 rotor at 3800 × g for 30 min in 250-ml Teflon bottles. The lipid/chloroform layer was separated from the floating protein disc by decanting into a round-bottom flask and the chloroform was removed on a rotary evaporator at a temperature less than 45°C. This procedure affords complete extraction of NAEs and other neutral lipids while minimizing the presence of glycolipids that interfere with further fractionation. The lipid extract was redissolved in 10 ml of chloroform and applied to a column of 30 g silicic acid (Davisil type 634) in a glass column 4 cm in diameter. Neutral lipids, including cholesterol, were eluted with 600 ml of chloroform and discarded. NAEs were eluted with 900 ml of 2% methanol in chloroform, collected in fractions of 100–150 ml each. The 2% methanol fractions were blown to dryness, redissolved in 1 ml of chloroform/methanol (2:1, v/v) and 50 μl of each was spotted on a TLC plate (silica gel H) and developed in hexane/diethylether/acetone/acetic acid (40:20:30:1, v/v/v/v). Fractions 3–6 contained material that had the same R_f as standard 18:1 NAE. These fractions were combined and NAE was isolated by TLC in the same solvent system; 18:1 NAE was spotted in a separate lane on one edge of the plate. The plate was sprayed with an ethanolic solution of 2′,7′-dichlorofluorescein, viewed under UV light and the region corresponding to the NAE standard was scraped off and eluted with diethyl ether/methanol (9:1, v/v) or with chloroform/methanol/water (30:50:20, v/v/v). When the latter solvent was used, chloroform and water were added to the eluate to achieve phase separation; the chloroform layer was recovered and taken to dryness under N_2.

2.4. Gas chromatography-mass spectrometry (GC-MS)

The isolated NAE fraction was reacted with 30 μl or 60 μl of *tert.*-butyldimethylsilyl (*t*-BDMS)/imidazole reagent (Alltech Associates, Deerfield, IL) at 80°C for 1 h. The *t*-BDMS ethers were injected directly into the GC-MS. GC-MS was done on a Hewlett-Packard 5890 Series II GC with a 5972 mass selective detector (MSD) and a 7673 automatic injector. The capillary column (HP5MS, 30 m × 0.25 mm i.d.) was temperature programmed from 90–270°C at 10°/min. The MSD was operated in selected ion monitoring mode measuring the M-57 ions 356/360, 384/388, 382/386 and 404/408 for 16:0, 18:0, 18:1 and 20:4 NAE, respectively. Calibration curves were linear, with the ion ratios equal to weight ratios for each chain length.

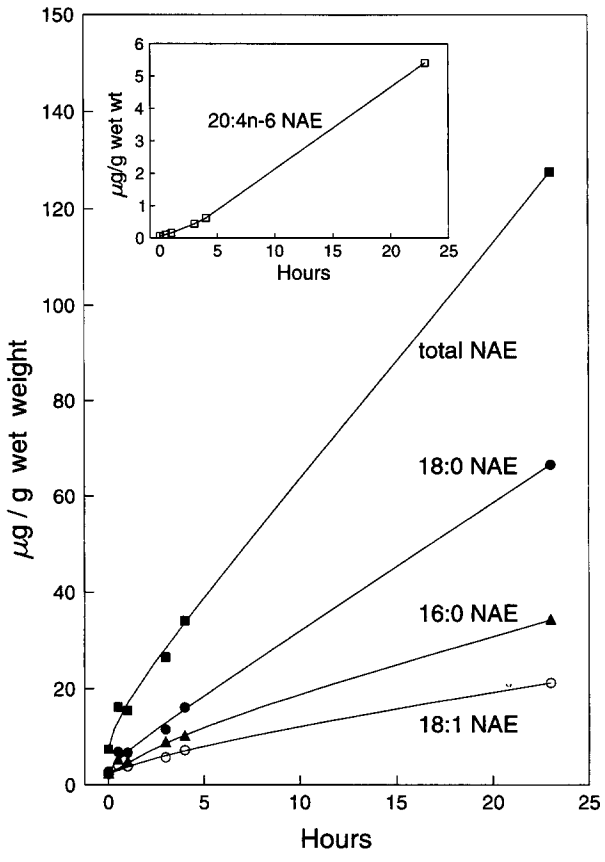

Fig. 1. Amounts (μg/g wet weight) of total NAE (■), 16:0 NAE (▲), 18:0 NAE (●), 18:1n-9 + 18:1n-7 NAE (○) and 20:4n-6 NAE (□, inset) in fresh pig brain kept at ambient temperature for 0, 0.5, 1, 3, 4 and 23 h. Lipids were extracted and analysed as described under section 2.

3. Results

We measured the endogenous levels of long-chain NAEs in mammalian brains that were either homogenized or frozen within 2–4 min after death. Frozen tissues were stored at −70°C and were cut up and homogenized in ice-cold chloroform immediately upon removal from the freezer. The values in Table 1, therefore, represent the best approximation of endogenous NAE concentrations in these brains.

Brains from pig, sheep and cow contained almost exclusively (~99%) saturated and monounsaturated NAEs, i.e. approximately equal proportions of 16:0, 18:0 and 18:1 NAE, the latter comprised of two positional isomers. About 1% of pig and cow brain NAE was identified as 20:4n-6 NAE (anandamide), whereas sheep brain did not contain measurable amounts of this compound. However, when portions of these brains that had been frozen immediately after death were thawed and kept at ambient temperature for 22 h, there occurred a dramatic increase in all NAEs and 20:4n-6 NAE could be detected in each brain (Table 2). Total NAE levels increased 37-, 33- and 72-fold, respectively, in pig, sheep and cow brain. In pig and sheep brain, the relative increase of 20:4n-6 NAE was greater than that of the other NAEs (see Table 1).

In order to better define the postmortem generation of NAE

Table 1
Amounts and composition of endogenous NAEs from the brains of pig, sheep and cow

	Pig[a]		Sheep[b]		Cow[b]	
	μg/g wet weight	%	μg/g wet weight	%	μg/g wet weight	%
16:0 NAE	2.05 ± 0.49	31.7	0.74, 0.76	28.2	1.11, 1.02	35.3
18:0 NAE	2.27 ± 0.43	35.1	0.87, 0.93	33.8	0.97, 0.86	30.2
18:1N-9 NAE	0.96 ± 0.17	14.8	0.27, 0.29	10.6	0.38, 0.32	11.5
18:1n-7 NAE	1.13 ± 0.14	17.5	0.74, 0.72	27.5	0.78, 0.55	21.9
20:4n-6 NAE	0.06 ± 0.01	0.9	0.0		0.04, 0.03	1.1
Total NAE	6.47 ± 1.14		2.63, 2.70		3.27, 2.77	

Brains were homogenized in chloroform in the presence of deuterated standards and NAEs were isolated and analysed as described under section 2.
[a] Mean ± S.D. from extracts of 5 brains, 3 fresh and 2 frozen.
[b] Analyses of 2 separate portions of 1 frozen brain.

Table 2
Amounts and composition of endogenous NAEs from portions of brains frozen, thawed and kept at ambient temperature for 22 h

	Pig[a]		Sheep[b]		Cow[b]	
	μg/g wet weight	%	μg/g wet weight	%	μg/g wet weight	%
16:0 NAE	82.3	33.9	41.2	46.2	81.8	37.9
18:0 NAE	91.1	37.6	29.1	32.6	85.4	39.5
18:1n-9 NAE	40.8	16.8	9.6	10.7	30.6	14.2
18:1n-7 NAE	23.0	9.5	5.9	6.6	16.2	7.5
20:4n-6 NAE	5.5	2.3	3.5	3.9	2.1	1.0
Total NAE[c]	242.6		89.3		216.1	

Extraction and assay as in Table 1.
[a] Averages from 2 brains analyzed separately.
[b] Averages of duplicate analyses from 1 brain.
[c] Small amounts of 18:2n-6 NAE were present in some extracts (tentatively identified without an internal standard).

in pig brain, we obtained 8 fresh brains and maintained portions of them (20 g each) in plastic bags at ambient temperature for 0.5, 1, 3, 4 and 23 h. Thereafter, NAE levels were assayed and the results are summarized in Fig. 1.

Total NAE levels increased almost linearly over the entire 23-h period with the greatest increase occurring in 18:0 NAE. Interestingly, the appearance of 20:4n-6 NAE exhibited a time lag compared with the other NAEs (Fig. 1, insert). This is probably related to the mechanism of its biosynthesis but remains unexplained at this time.

4. Discussion

We report here for the first time the endogenous levels and compositions of long-chain *N*-acylethanolamines of mammalian brain. Because these levels rise rapidly postmortem and this can lead to the generation of NAEs which are not originally present in detectable amounts (e.g. 20:4n-6 NAE in sheep brain), we recommend that future reports should indicate the history of the neural tissue analysed. Quantification using a deuterated internal standard for each NAE is the method of choice because of the very low levels found in brain, the potential for significant losses of NAE during isolation from the bulk of the brain lipids, and the differential response of the MSD to saturated vs. unsaturated compounds. The preferred derivatives for GC-MS are *tert.*-butyldimethylsilyl ethers because they separate well on the non-polar capillary column used and produce a reliable M-57 ion for each chain length.

Our present results raise intriguing questions regarding the biosynthesis of long-chain *N*-acylethanolamines in brain. It is likely that the saturated and monounsaturated NAEs are generated by the transacylation-phosphodiesterase pathway elaborated by us [22–29], a reaction sequence that we have previously shown to produce *N*-acylethanolamine phospholipids in postmortem rat brain [19]. Recent evidence suggests that *N*-acylethanolamine phospholipids having polyunsaturated *N*-acyl moieties can also be produced in cultured brain neurons [31] and could thus serve as precursors of polyunsaturated NAEs. This biosynthetic pathway would require at least a transient presence of arachidonate at the *sn*-1 position of glycerophospholipids which could occur through de novo synthesis of diarachidonoyl molecular species [32]. Alternatively, polyunsaturated NAEs, including 20:4n-6 NAE, could be formed by direct condensation of the fatty acids with ethanolamine [13,14]. This reaction exhibits a high K_m (20–100 mM) for ethanolamine and thus may represent the reverse reaction of amidohydrolase activity that has been shown to degrade and synthesize saturated and monounsaturated NAEs [11]. It is interesting to note, however, that an amidohydrolase highly selective for 20:4n-6 NAE was recently identified in regions of the brain rich in cannabinoid receptors [15]. Further work will be aimed at explaining the different kinetics for the postmortem generation of saturated-monounsaturated NAEs vs. the polyunsaturated 20:4n-6 NAE (Fig. 1) and to determine the biosynthetic processes involved.

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