Targeted Lipidomics: Discovery of New Fatty Acyl Amides

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ABSTRACT

The discovery of endogenous fatty acyl amides such as *N*-arachidonoyl ethanolamide (anandamide), *N*-oleoyl ethanolamide (OEA), and *N*-arachidonoyl dopamine (NADA) as important signaling molecules in the central and peripheral nervous system has led us to pursue other unidentified signaling molecules. Until recently, technical challenges, particularly those associated with lipid purification and chemical analysis, have hindered the identification of low abundance signaling lipids. Improvements in chromatography and mass spectrometry (MS) such as miniaturization of high-performance liquid chromatography components, hybridization of multistage mass spectrometers and time-of-flight technology, the development of electrospray ionization (ESI) and of information-dependent acquisition, now permit rapid identification of novel, low abundance, signaling lipids.

KEYWORDS: lipidomics, cannabinoid, fatty acyl amide, information-dependent acquisition, HPLC-MS/MS, nano-HPLC

INTRODUCTION

Since the early 1990s several endogenous lipid derivatives such as anandamide,¹ 2-arachidonoyl glycerol (2-AG),² *N*-arachidonoyl dopamine (NADA),³ and *N*-arachidonoyl glycine (NAGly)⁴ have been found in rodent and bovine brains, and their biological actions⁵ and signal transduction pathways have been rigorously studied.⁶⁻⁸ It is now well established that these fatty acyl amides and esters modulate several physiological processes, including pain sensitivity,⁹ reproduction,¹⁰ immune function,¹¹ and vascular tone¹² among others.

FATTY ACYL AMIDES AS SIGNALING MOLECULES

Anandamide was isolated from porcine brains using column chromatography and thin layer chromatography (TLC), and

¹H nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectra were identical to the synthetic material.¹ The physiological functions of anandamide are similar to those of Δ^9 -tetrahydrocannabinol (Δ^9 -THC) in that both activate the cannabinoid receptor 1 (CB1),¹³ and anandamide also acts on the transient receptor potential vanilloid 1 (TRPV1).¹⁴ Homo- γ -linolenoyl ethanolamide and docosahexaenoyl ethanolamide were partially purified from porcine brains on a silica gel column and identified by comparison of the gas chromatography (GC)-MS spectra of the endogenous compounds with those of the synthetic compounds.¹⁵ Palmitoyl ethanolamide (PEA) was originally isolated from soybeans, egg yolk, and peanut meal,¹⁶ and subsequently from mammalian tissue.¹⁷ The structural information was obtained by infrared spectroscopy.¹⁶ Although PEA does not bind the cannabinoid receptors 1 and 2 (CB2) with high affinity, it enhances the effect of anandamide by an unknown mechanism.¹⁸ Oleoyl ethanolamide (OEA) was purified using 3 high-performance liquid chromatography (HPLC) columns and identified by GC-MS.¹⁹ OEA activates the peroxisome proliferator-activated receptor α (PPAR- α)^{20,21} and TRPV1 receptors,²² while it lacks high affinity for CB1 and CB2 receptors.^{20,21}

NADA and other fatty acyl dopamines were identified by our laboratory³ using solid phase extraction and HPLC-MS/MS. They are found in nervous tissues, especially striatum, hippocampus, cerebellum, and dorsal root ganglion. NADA activates TRPV1 receptors with a potency similar to that of capsaicin; it also binds to CB1 and shows cannabimimetic effects.³ *N*-oleoyldopamine (OLDA) is more potent than NADA at TRPV1 and less potent at CB1.²³ The dopamines of palmitic and stearic acids lack affinity for TRPV1 and CB1 but act in concert with NADA and anandamide to enhance TRPV1-mediated calcium mobilization.²⁴ These findings led to the hypothesis that synthesis and release of NADA and OLDA would cause pain sensations via activation of TRPV1.

NAGly was synthesized for structure-activity studies of the CB1 receptor,²⁵ and our laboratory reported the existence of NAGly as a natural constituent of brain, along with *N*-arachidonoyl- γ -aminobutyric acid and *N*-arachidonoyl alanine.⁴ These were the first endogenous fatty acyl amino acids identified in mammals. In these studies, Huang et al⁴

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used ion exchange and reversed phase solid phase extraction to partially purify the compounds prior to liquid chromatography (LC)-MS/MS on the ion trap and hybrid quadrupole/time-of-flight (QqTOF) instruments. Widely distributed among mammalian tissues, NAGly causes hot plate analgesia in mice and suppresses formalin-induced pain behavior in rats.⁴ Recently, *N*-arachidonoyl serine was identified in a bovine brain extract by Milman et al.²⁶ This compound triggers vasodilatation of rat mesenteric arteries and abdominal aorta. These findings suggest that other fatty acyl amino acids with similar structures may also exist endogenously and possess similar biological functions.

CHALLENGE OF LIPID IDENTIFICATION AND RESOLUTION

From the last section, it is clear that there are 2 types of identification strategies used for fatty acyl amides. In the first, extracts are purified to homogeneity and subjected to NMR, infrared (IR), and MS analysis. This approach works well with compounds that are abundant in biological systems but not for those that occur at trace levels. Milligram quantities of compound in pure form are required for ¹³C NMR and microgram quantities for ¹H NMR. This requirement is extremely difficult for compounds that occur in the pmol/g range (eg, anandamide, NAGly), requiring the use of tissue from large species such as cow or pig, and even in these cases, the amounts that can be isolated often do not allow for ¹³C NMR.¹

A second method relies on HPLC-MS/MS methods, where accurate masses are obtained (<10 ppm) and the full fragmentation pattern and HPLC retention time of endogenous material is compared with that of synthetic material. While it is still possible for different chemical species to produce nearly identical mass spectra, high precision mass measurements, coupled with appropriate fragmentation patterns and retention times allow the proposal of the chemical formulae and structures with a reasonable degree of confidence. The amount and the purity of the sample are not as critical as in the first method because recent advances in MS and liquid chromatography permit isolation of molecular species of a particular molecular weight (unit accuracy) followed by product ion scans with trace amounts (eg, 250 fmol on column). Using this approach, small amounts of tissue (eg, 2-6 g) are subjected to a series of liquid-liquid extraction and solid-phase purification, and a QqTOF instrument coupled with nano-HPLC is used to identify the compound.

Whereas the QqTOF performs well for identification of novel compounds, multiple-reaction monitoring (MRM) scan on a triple quadrupole MS coupled with HPLC is the preferred method for quantifying levels of endogenous compounds. This method is useful for pilot studies because the automation of the triple quadrupole with HPLC makes

MRM extremely sensitive and easy to use. Although such experiments often yield a single peak, matching of retention times to a standard is required for compound identification. However, MRM experiments cannot replace product ion scans because only one or a few major fragment ions are used in MRM experiments, while a full range mass spectrum is required for identification of new compounds. Although a product ion scan can be performed on a triple quadrupole MS, it requires considerably more (10- to 50-fold) sample and produces reduced mass resolution and accuracy compared with the QqTOF.

Large increases in sensitivity can be obtained using nano-HPLC because the MS signal is proportional to the concentration of the sample, and the smaller column (75 μ m inner diameter [id]) requires less sample to produce the same concentration than standard analytical (4.1 mm id) and narrow bore (2.1 mm) columns. Here, the sensitivity increases by the second power of the column diameters with a similar decrease in flow rate. Hence, lowering the column diameter from 4.1 mm to 75 μ m requires a drop in the flow rate from 1 mL/min to ~250 nL/min, with a concomitant theoretical increase in sensitivity of 1600-fold. Although the theoretical increase in sensitivity is not achieved in our laboratory, when coupled with nano-HPLC, typically less than 250 fmol of a fatty acyl amide on column is required in the product ion scan mode for QqTOF for identification of new compounds and 100 attomol is required in the MRM scan on the triple quadrupole MS for quantification of known compounds. The relatively recent development of nano-HPLC, triple quadrupole, and TOF mass spectrometers provides investigators with markedly improved methods for identification of low abundance signaling lipids.

INFORMATION-DEPENDENT ACQUISITION

Although lipids present in the cell differ greatly in their structural, chemical, and physical properties, they typically occur in families composed of conjugates of lipid moieties and functional groups in a combinatorial fashion. This was evident, for example, in the various fatty acyl ethanolamides, of which anandamide is but one product, and a minor one.²⁷ Precursor ion and neutral loss scans available with the triple quadrupole MS are the common tool to identify a group of lipids with similar structures. These scanning modes require relatively large amount of samples compared with MRM, which is the bottleneck for identification of trace lipids. To improve throughput for proteomics, a method was needed to identify as many putative compounds as possible in one run, while maintaining high sensitivity. Information-dependent (or data-dependent) acquisition (IDA)²⁸ was developed to provide this capability. Currently, IDA methods are typically available for QqTOF, triple quadrupole, and ion trap mass spectrometers. No baseline separation of analytes is required,

and the mass accuracy and sensitivity is not compromised.²⁹ With appropriate improvements in offline data analysis, IDA has proven useful for lipidomic approaches.³⁰

A cycle of IDA contains a survey scan and several corresponding MS/MS events. The general mechanism is shown in Figure 1. Ions found in the survey scan are examined using a series of switch criteria. The computer selects ions for further experimentation based upon such factors as intensity, presence in an inclusion list, absence from an exclusion list, recency of occurrence, isotope ratio, and a fixed mass difference between pairs of ions. A full scan without fragmentation of the molecular ion is the most common survey scan but neutral loss, precursor ion, and MRM scans can also trigger the product ion scan.

As noted above, IDA was developed for and is widely used in proteomics and metabolomics research. The authors are aware of only one report on the use of IDA for lipidomics.³⁰ In that report, IDA was used to identify 35 known triacylglycerols and 90 glycerophospholipids in a lipid extract from *Caenorhabditis elegans*.³⁰ While it was possible to identify a large number of high abundance lipids by this approach, the use of infusion rather than an HPLC separation would greatly limit the number of signaling lipids that could be identified. Detection of compounds within the nanomolar concentration range was based on using 20 to 30 seconds as the accumulation time. This approach would not be practical with HPLC separation because only one ion per LC peak would be possible, thereby eliminating analysis of coeluting lipids. The authors' suggestion for analysis of low abundance compounds was to use an inclusion list. While a logical step, this presupposes the structure of the compound to be studied, and it should be noted that the peak intensities obtained with HPLC would be much greater, relative to background noise, than that obtained with flow injection. Hence, the detection limits of low abundance compounds would still be much lower with flow injection than with LC separation.

We developed a sensitive IDA method that was successfully applied to identification of new lipids in a biological sample.

Figure 1. Scheme of a cycle of IDA.

Methanol extracts of rodent brains were purified by solid phase extraction on ion exchange, reversed phase, and normal phase columns, and the fractions were injected into a nano-LC/hybrid quadrupole/TOF MS using nano-electrospray ionization (Applied Biosystems/MDS Sciex QStar, Foster City, CA) at a flow rate of 250 nL/min. Data were acquired with IDA triggered by TOF masses obtained in Analyst Software (Applied Biosystems/MDS Sciex). The previously selected target ions were excluded for 30 seconds. Isotope ions were excluded throughout the run, and none of the inclusion and exclusion list was used. One TOF scan and the following MS/MS scan from one IDA run are shown in Figure 2. The data were analyzed with a computer program constructed in house, which compared spectral peaks from the MS with theoretical masses based on the formulae combinatorially of 15 fatty acids and 23 amino acids and other small endogenous amines capable of forming amides. An initial match required the spectra from the IDA experiment to contain the mass of the molecular ion and a fragment mass corresponding to the amino acid.

Figure 2. Total ion count chromatogram from lipid brain extract from nano-LC to QqTOC: (A) TOF scan of a rat brain extract using nano-HPLC/MS/MS (75 μ m column, 250 nL/min); (B) one product ion scan triggered by TOF scan contains the mass of the molecular ion (*N*-arachidonoyl taurine m/z 412.2409) and a fragment mass corresponding to taurine (m/z 126.0136). This confirms the finding of *N*-arachidonoyl taurine by Saghatelian et al.³¹

Subsequent inspection of matches was performed to confirm the presence of other predicted fragments. The MS/MS spectra of the brain extract were then compared with a library of synthetic *N*-acyl amino acids (>100). The results obtained from the computer program were consistent with those obtained from inspection of the mass spectra. One run of an IDA experiment generated ~1000 mass spectra from which more than 40 acyl amino acids were identified. Of these, more than 30 were novel compounds and had structures similar to previously identified fatty acyl amides. It is possible that these novel endogenous compounds have important biological signaling functions.

SUMMARY

Mass spectrometric approaches to the identification of lipid signaling molecules are reviewed here. Compared with proteomics, there are fewer applications of lipidomics, indicating difficulties associated with the identification of endogenous lipids. Specific purification and HPLC/MS-MS methods have been designed and proved highly efficient to identify the members of one family of lipids, the fatty acyl amides. Some members of this family have demonstrated important biological functions and play a role in pain, immune function, reproduction, and appetite.

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