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Gene expression of NAPE phospholipase D enzyme and its role in hormonal regulation

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ABSTRACT:
N-acylethanolamines (NAEs) are membrane-derived lipids that are utilised as signaling molecules in the nervous system and elsewhere in the body. A *N*-Acylphosphatidylethanolamine Phospholipase D (NAPE-PLD) that catalyses formation of NAEs has been identified as a member of the zinc metallohydrolase family of enzymes. Intense NAPE-PLD immunoreactivity was also detected in axons of the vomeronasal nerve that project to the accessory olfactory bulb. NAPE-PLD expression was detected in other brain regions (e.g. hippocampus, cortex, thalamus, hypothalamus), but the intensity of immunostaining was weaker than in mossy fibres. Collectively, the data obtained indicate that NAPE-PLD is expressed by specific tissues in the body. Specific gene knock out animals have also been created. Recent cDNA cloning of the NAPE-hydrolyzing PLD (NAPEPLD) from mouse, rat and human revealed that NAPE-PLD is a novel enzyme which has no homology with any known PLD enzymes, but belongs to the zinc metallo-hydrolase family of the β-lactamase fold. The recombinant enzyme hydrolyzed various NAPEs, including the anandamide precursor N-arachidonoylphosphatidylethanolamine at similar rates, but was inactive with phosphatidylcholine and phosphatidylethanolamine. Considering cannabimimetic activities of anandamide, the enzymes involved in the biosynthesis and degradation of anandamide, including NAPE-PLD, may be promising targets for therapeutic agents.
Key words: NAPE, phospholipase D, Gene, expression, hormone, metabolism

INTRODUCTION:

The discovery of anandamide as an endocannabinoid has stimulated interest in the mechanisms of biosynthesis of NAEs in mammals. NAE biosynthesis has been proposed to occur via a two-step enzymatic process. First, a Ca^{2+}-activated Nacyltransferase transfers the sn-1 acyl chain of a phospholipid onto the amine of phosphatidylethanolamine (PE) to generate an *N*-acyl PE (NAPE). Then NAPE is converted by a phospholipase D (PLD) into an NAE and phosphatidic acid (1-3). NAPE-PLD is a 396 amino-acid residue protein in mouse and rat and a member of the zinc metallohydrolase family with a lactamase fold (Fig.3). Analysis of the occurrence of NAPE-PLD mRNA/protein in mouse organs/tissues revealed widespread expression but with particularly high levels in brain and testis, consistent with the distribution of NAPE-PLD enzymatic activity.“knockout” mice with the NAPE-PLD gene deleted have been generated to investigate the *in vivo* contribution of NAPE-PLD to NAE biosynthesis (4).The sequence of

the NAPE PLD gene predicts a member of the metallo-b-lactamases (MbLs) (5), a protein superfamily that includes hydrolases involved in antibiotic resistance, DNA repair, and RNA maturation (6,7). NAPE-PLD is unrelated to other mammalian phospholipases D (PLD1 and PLD2) (8) and further differs from these enzymes in its ability to selectively hydrolyze NAPEs (9). Importantly, NAPE PLD recognizes all NAPEs, irrespective of their N-acyl substituents (10), and is therefore able to produce FAEs with diverse biological activities.Elucidation of gene expression of NAPE phospholipase D will help in the understanding of functional role in patho-physiological states.This review article is focussed on the gene expression of NAPE phospholipase D and its role in endocrine function.

Immunocytochemistry:

Immunocytochemical analysis of NAPE-PLD expression can be performed using brains from mice of the BALB/c strain to establish methodology prior to analysis of brains from male NAPE-PLD-knockout mice (NAPE-PLD-/-) and male wild-type littermates (NAPE-PLD+/+). Two separate methods for preparation of brain sections are tested: 1. Sectioning of paraffin-wax embedded brains fixed with 4% PFA in PBS and post-fixed in Bouin's fixative, as described previously (11) or 2. Sectioning of frozen brains fixed with 4% PFA in PBS. The later method is found to be preferable for immunocytochemical visualisation of NAPE-PLD expression usingthe antibodies developed in this study and therefore this method is described. Sections are then incubated overnight at 4^{o}C with affinity-purified antibodies to mouseNAPE-PLD (2282.3T) diluted 1:7 - 1:10 in PBSTx with 5% NGS and 0.05% sodium azide added. After washing in PBSTx, slides are incubated for 4 h at room temperature with horseradish-peroxidase conjugated goat anti-rabbit immunoglobulins (Jackson ImmunoResearch). Then after washing in PBS, bound antibodies are revealed using diaminobenzidine with nickel as a substrate (Vector Labs. Inc., Burlingame, CA 94010, USA). Finally, free-floating sections were collected on polysine slides (VWR),dehydrated, cleared in xylene and prepared for microscopy by mounting coverslips over DPX. Images of stained sections were then captured as described above for mRNA *in situ* hybridization (12).

Gene expression of NAPE phospholipase D in rat brain:

NAPE-PLD mRNA has been detected in the somata of identified neuronal populations in several regions of the brain, including granule cells in the dentate gyrus, olfactory bulb, and cerebellar cortex and pyramidal cells in the hippocampus and cortex.Importantly, the overall regional distribution of NAPE-PLD mRNA in mouse brain correlates with the distribution of NAPE-PLD protein as determimed by Western blotting (Fig.1). For example, both NAPE-PLD mRNA and protein are very abundant in the hippocampal formation but are present at relatively low levels in the cerebellum. Previously, the relative abundance of NAPE-PLD in different regions of the rat brain has been investigated by measurement of NAPE-PLD enzyme activity, Western blotting and real time PCR (13). NAPE-PLD was detected in all rat brain regions analysed but it was most abundant in the thalamus. These data also indicate that NAPE-PLD is widely expressed in thalamus, but based on the intensity of immunostaining it appears that in mouse brain NAPE-PLD is more abundant in the hippocampal formation. Now whether this has implications for diseases like the Parkinson or Muscular dystrophy needs to investigated. There may therefore be species differences in the relative abundance of NAPE-PLD in brain regions.LPS altered the acetylation state of histone proteins bound to the NAPE-PLD promoter and suppressed the transcription of NAPE-PLD mRNA (14).The

transcription factor Sp1 was involved in the regulation of baseline NAPE-PLD expression, but not in the suppression by LPS. In addition, the expression of NAPE-PLD in rodent brains is age-dependently upregulated at the mRNA and protein levels (15), in agreement with the increase in NAPE-PLD activity (16-17). In contrast, N-acyltransferase activity decreases duringdevelopment (18). The opposite changes of these two enzymes probably explain why the NAPE level in the ischemic brain of young rodents is much higher than that in adult rodents. NAPE-PLD_/_mice were born at the expected Mendelian frequency, were viable, and were apparently healthy (19-20). The accumulation of NAPEs in thebrains of NAPE-PLD_/_mice demonstrated an important role of NAPE-PLD in the degradation of NAPE in this tissue. However, the detection of unaltered or moderately reduced NAE levels stronglysuggested the existence of other NAE-forming enzyme(s) or route(s) in NAPE-PLD_/_mice. Apart from this gene-modified mouse model, a recent cohort study suggested a physiological role of NAPE-PLD. In this study, a common haplotype in NAPE-PLD was reported to be protective against severe obesity for e.g in a Norwegian population (21).

Gene knock out animal models:

In the first line of NAPE-PLD KO mice, developed in the laboratory of Ben Cravatt (referred to as the Cravatt line), no differences were found in whole-brain levels of AEA compared to wild type mice (WT), despite the fact that brain homogenates from KO mice showed lower activity in synthesizing AEA from exogenously added NAPE. Neither did they find any difference in levels of *N*-docosahexaenoyl ethanolamine (DEA). Changes in NAE production were restricted to saturated and monounsaturated fatty acid conjugates, with significant decreases in *N*-palmitoyl ethanolamine (PEA), *N*-stearoyl ethanolamine (SEA), and *N*-oleoyl ethanolamine (OEA). In this line of NAPE-PLD KO mice, the larger decreases in NAE levels were in 20, 22 and 24 carbon saturated and monounsaturated fatty acid conjugates. *N*-linoleoyl ethanolamine (LEA) levels were not measured in the Cravatt line (22).An additional strain of NAPE-PLD KO mice was later developed in the laboratory of Dale Deutsch (referred to as the Deutsch line). In this strain of NAPE-PLD KO mice, whole brain levels of AEA were significantly lower than WT. Additionally, levels of NAEs containing unsaturated conjugates, such as OEA, LEA, and DEA, were like wise significantly lower in the NAPE-PLD KO mice. Results were more varied for saturated conjugates, as these NAPE-PLD KO mice had significantly lower levels of PEA, whereas levels of SEA were not significantly different from WT mice. Additionally, the concentration of longer chain saturated NAEs with 20 and 22 carbons were not statistically different from WT in the NAPE-PLD KO mice (23-24).A third NAPE-PLD KO mouse was developed by Serge Luquet when he was a post-doctoral researcher in Richard Palmiter's lab (referred to as the Luquet line) wherein brain homogenates from the Luquet line showed reduced conversion of exogenous NAPE to AEA by about 75% compared to WT (25). However, lipid levels have not yet been measured in the Luquet line of NAPE-PLD KO mice.

Technique for mRNA extraction of NAPE phospholipase D and PCR analysis:

This technique has been nicely described by Chenggang Zhu *et al* in a article published in 2011 (26). The total RNA using Trizol TM (Invitrogen) cDNA was synthesized with 0.2 µg of total RNA and oligo (dT)12-18 primer using Superscript II RNase H-reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). They designed primer/probe sets using the Primer Express TM software based on gene sequences available from the GenBank TM database. Primers and fluorogenic probes were

synthesized at TIB (Adelphia, NJ). The primer/probe sequences for mouse genes were as follows. NAPE phospholipase D: forward: 5'- MOL #702017 AACGAGCGGTTCGGCA-3', reverse: 5' ATCCAGTCAAGAAGGCCCAA-3'; probe: 5'-CGAGCTGCGGTGGTTTGTGCC-3'. Naaa: forward: 5'CGGTGGCGCAGGTCA-3', reverse: 5'-AATTTCTCCGACCATCCCG-3'; probe: 5'-TGGCGACAGGGTTCCCCAGTG-3'. Faah: forward: 5'-CCTTATGCCCTGGAGGTCCT-3', reverse: 5'-GGAGAAAAGAGCAGCCACC A-3'; probe: 5'-TCGGCAGGTGGGCTGTTCAGTGT-3'. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh):forward: 5'-TCACTGGCATGGCCTTCC-3', reverse: 5'-GGCGGCACGTCAGATCC-3'; probe: 5'-TTCCTACCCCCAATGTGTCCGTCG-3'. RNA levels were normalized using the GAPDH as an internal standard.

NAPE phospholipase D and endocrinal function:

NAPE PLD enzyme seems to be the target of addictive drugs which may cause dysphoria or euphoria by altering the levels of anandamide in the brain and the spinal chord. Bliss of happiness may be the direct effect of anandamide levels in discrete brain sites. It appears that it may also affect the synthesis and metabolism of adrenal steroids by modulating stress and physical fatigue. Thus NAPE phospholipase D activity does seem to play an important role in physiological homeostasis. As far as the effects on the endocrine hormones there are several articles documenting its modulatory and salutary effects on endocrine hormones.The most potent growth stimulating oestrogen is 17β-oestradiol (E2), which has been linked directly and indirectly with the endocannabinoid system, where E2 stimulates NAPE-PLD and inhibits Fatty acid amide hydrolase (FAAH) synthesis and directly stimulates the release of AEA from endothelial cells (27-28). By contrast, another study revealed that NAPE-PLDis downregulated in the uterus by oestradiol, suggesting that it results in decreased anandamide levels, although this was not directly tested [27]. Progesterone has also been documented to downregulate uterine NAPE-PLD expression in mice, leading to a decrease in tissue AEA levels [27]. In the pregnant mouse uterus, it has also been reported to downregulate FAAH activity [28], and when taken together with reduced NAPE-PLD expression in mice these data suggest that the NAPE-PLD: FAAH activity ratio in the mouse uterus may be key to the regulation of local AEA levels and thus maintenance of pregnancy or endometrial pathologies, such as cancer. Anandamide a product of the effects of NAPE phospholipase D may also contribute in reproduction. Anandamide has been reported to have pleiotropic effects on reproduction; however, the mechanism by which it exerts these effects is unclear. Higher AEA concentrations were found in the placenta (29), and significant changes in AEA levels have been detected at the end of pregnancy in maternal blood (30), suggesting that the endocannabinoid system could modulate physiological functions during pregnancy and labor.The researchers delivered NAPEs and NAEs intestinally to mice using gut bacteria they developed that synthesize the compounds. They found that in mice missing the NAPE-PLD enzyme, increasing intestinal levels of NAPEs failed to reduce food intake and weight gain or alter gene expression, while increasing intestinal levels of NAEs still induced all of these effects. The findings, reported in the Journal of Lipid Research, show that NAPE-PLD activity is required for the leptin-like effects of NAPEs. Reduced NAPE-PLD activity reported in obese subjects may directly contribute to excess food intake and obesity. There is intensive research being conducted on the novel NAPE phospholipase D inhibitors at various centres and the laboratory of Dr.Piomelli has conducted some pioneering research on this topic (31-32).

NAPE phospholipase D gene: NAPE phospholipase D has been cloned from mouse, rat and human and 393-396 amino acid in length, with an estimated molecular wt. of 46 kDA.Both NAPE-PLD mRNA and protein activity have been shown to be expressed in a wide range of tissues with the highest levels in the brain, kidney and Testis (Fig.2). In rat, NAPE phospholipase D activity in the brain is low in neonates and is 15 fold higher in adults, where as the activity remains constant in the heart during development (15). NAPE-PLD expression in leukocytes is controlled by two distinct transcriptional mechanisms. Under unstimulated conditions, NAPE-PLD expression is maintained through a steady-state level of histone acetylation, which renders the NAPE-PLD gene accessible to transcriptional regulators such as Sp1. When stimulated with LPS, macrophages initiate a molecular cascade that results in the transcriptional suppression of NAPE-PLD, which is associated with decreased acetylation of histone proteins bound to the NAPE-PLD promoter. This model is supported by the effects of the HDAC inhibitor, trichostatin A, which blocks the transcriptional control of NAPE-PLD expression by LPS and concomitantly increases histone acetylation. Further experiments are needed to identify the effectors responsible for mediating histone deacetylation at the NAPE-PLD promoter, as well as the histones involved in this response.

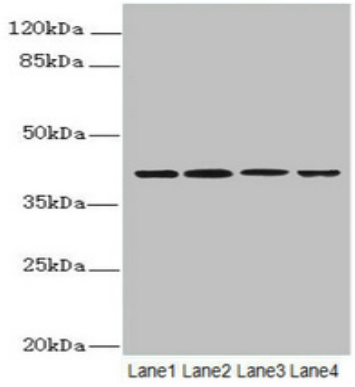

Fig.1: Western blotting image using a polyclonal antibody to NAPE phospholipase enzyme (Courtesy: My Biosource.com, Canada)

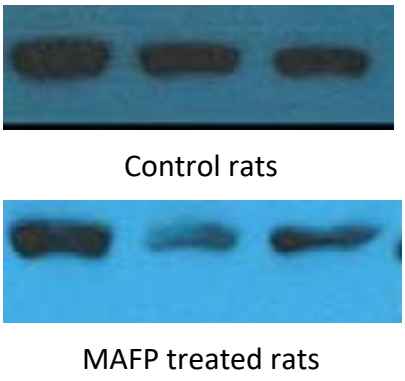

Fig.2: The NAPE phospholipase D mRNA activity in control and Methyl arachidonyl fluorophosphonate (MAFP, 20µM) treated rats

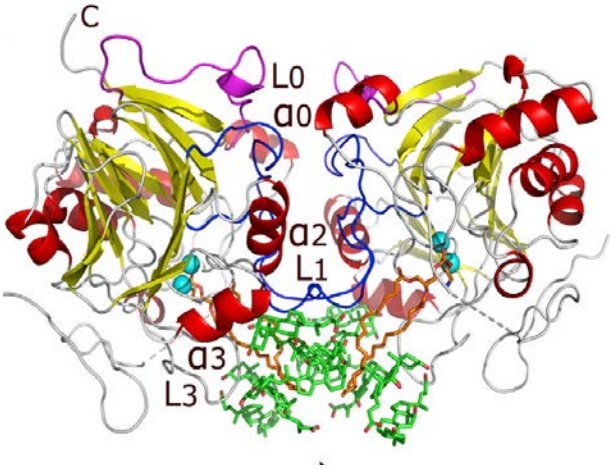

Fig.3: Ribbons diagram of the NAPE phospholipase D enzyme (Courtesy: P Magotti *et al*., 2015, Structure 23, 598–604)

Conclusion:

NAPE-PLD is a key enzyme in the synthesis of phosphatidic acid and Anandamide. In the brain,NAPE-PLD is hypothesized to be responsible for the production of NAEs, yet, to rule out that it is involved in broader areas of lipid metabolism a wider variety of lipids must be screened in more discrete brain regions. In a targeted lipidomics screening of over 70 N-acyl amides illustrates that in the Luquet NAPE-PLD KO mouse not only were there significant decreases in levels of NAEs throughout the brain, they were fatty acid chain and area dependent. Levels of other N-acyl amides, 2-acyl glycerols and prostaglandins were also significantly impacted by NAPE-PLD deletion in

a region-specific manner. This was the first report of changes in prostaglandin production in any NAPE-PLD KO mice. Therefore, deletion of NAPE-PLD impacts production of several cannabimimetic molecules, and is not just important for the synthesis of NAEs. Furthermore, the impact of NAPE-PLD deletion on the lipidome varies by brain region, which no doubt has functional implications. The widespread lipidomics shifts identified in the Luquet NAPE-PLD KO mouse highlights the interconnectedness of the arachidonic acid lipidome and may explain some of the behavioral changes seen after deletion of this enzyme. This enzyme needs to be further investigated for possible role in neurodegenerative disorders.

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