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Studies on the anorectic effect of *N*-acylphosphatidylethanolamine and phosphatidylethanolamine in mice

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ABSTRACT

N-acyl-phosphatidylethanolamine is a precursor phospholipid for anandamide, oleoylethanolamide, and other *N*-acylethanolamines, and it may in itself have biological functions in cell membranes. Recently, *N*-palmitoyl-phosphatidylethanolamine (NAPE) has been reported to function as an anorectic hormone secreted from the gut and acting on the brain (Gillum et al., [5]). In the current study, two of our laboratories independently investigated whether NAPE metabolites may be involved in mediating the anorectic action of NAPE *i.p.* injected in mice. Thus, the anorectic activity of a non-hydrolysable NAPE analogue, having ether bonds instead of ester bonds at *sn*1 and *sn*2 was compared with that of NAPE in molar equivalent doses. Furthermore, the anorectic effect of NAPE in NAPE-hydrolysing phospholipase D knockout animals was investigated. As negative controls, the NAPE precursor phosphatidylethanolamine and the related phospholipids phosphatidylcholine and phosphatidic acid were also tested. All compounds except one were found to inhibit food intake, raising the possibility that the effect of NAPE is non-specific.

1. Introduction

The gastrointestinal tract is continuously sending signals to the brain about the type and amount of nutrients in the lumen, and these signals take part in regulating food intake [18]. The signals can be hormonal, *e.g.* ghrelin, CCK, GLP-1 and GIP, or be neuronal via the vagus nerve [7]. Recently it was discovered that locally acting lipids, oleoylethanolamide, palmitoylethanolamide and linoleoylethanolamide, act as signalling molecules [2,4,15] that may serve as a satiety signal through the nuclear receptor PPARalpha, and the vagus nerve to the brain [2,3]. They belong to the family of *N*-acylethanolamines (NAE) [8], and their intestinal levels in rats are decreased by an increased intake of dietary fat [1,2]. These anorectic lipids are formed in the intestine from the precursor phospholipids, *N*-acyl-phosphatidylethanolamines (NAPE), via one or more enzymatic pathways [8,24]. One possible pathway is via the NAPE-hydrolysing phospholipase D (NAPE-PLD) [14] that generates NAE plus phosphatidic acid (PA). Another pathway has also been described, in which a serine hydrolase, α/β-hydrolase 4 (Abh4) cleaves of the two fatty acids in the *sn*1 and *sn*2 positions of NAPE, before glycerophosphodiesterase-1 (GDE1) generates NAE (Fig. 1) [20,21]. NAPE, in turn, is formed by transfer of a fatty acid from a donor phospholipid, *e.g.* phosphatidylcholine (PC), to phosphatidylethanolamine (PE) by the membrane-bound enzyme *N*-acyltransferase [24]. NAPE can be found in small amounts (pmol/g tissue) in all mammalian tissues [16]. Besides being a precursor for NAE, the physiological role of NAPE is not clear, but it will accumulate during cell injury [9] and it clearly influences membrane organization [12,22,23] and may have anti-inflammatory activity in macrophages [19]. Recently, NAPE (containing a *N*-palmitoyl group) has also been suggested to function as an anorectic hormone that is secreted from the intestine with the chylomicrons and enters the brain to exert its anorectic effect [5]. Phospholipids can function as signalling molecules, e.g. phosphatidylserine in the outer leaflet of the cell membrane [25], and different inositol phospholipids in membranes

Abbreviations: NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; PLD, phospholipase D; PA, phosphatidic acid; Abh4, α/β-hydrolase 4; GDE1, glycerophosphodiesterase-1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; De-NAPE, 1,2-di-hexadecyl-*sn*-glycero-3-phospho-*N*-palmitoylethanolamine; NAPE-PLD$^{-/-}$, NAPE-PLD knockout animals; DMAP, dimethylaminopyridin; *i.p.*, intraperitoneally

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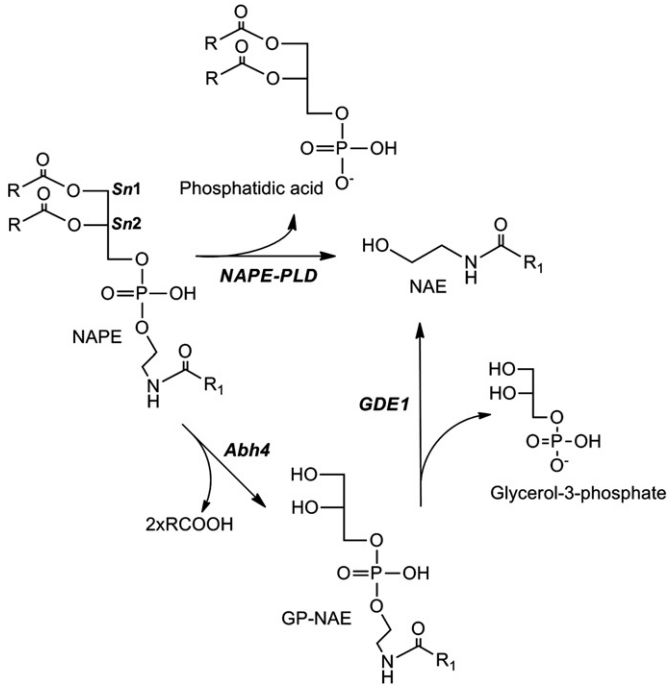

Fig. 1. Overview of NAPE metabolism. NAPE is metabolised to NAE via two different pathways. NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NAPE-PLD, NAPE-hydrolysing phospholipase D; Abh4, α/β-hydrolase 4; GDE1, Glycerophosphodiesterase-1.

facing the cytosol [10], but it is unusual that such a large lipophilic molecule as NAPE should by itself have a signalling function outside the cell. Lysophospholipids are known to bind and stimulate G-protein coupled receptors [11,13], but lysophospholipids are considerably more hydrophilic than NAPE. Two of our research groups independently hypothesised that the anorectic effect of NAPE could be mediated by a NAPE metabolite, either generated by hydrolysis of the *O*-acylated fatty acids, forming the lysophospholipid-like glycerophospho-*N*-acylethanolamine, or generated by hydrolysis of the phospho-ester bond by NAPE-PLD, *e.g.* PA or *N*-palmitoylethanolamine. We have tested the previously described anorectic effect of a NAPE species [5], 1,2-di-palmitoyl-*sn*-glycero-3-phospho-*N*-palmitoylethanolamine, compared with a non-hydrolysable di-ether derivative (1,2-dihexadecyl-*sn*-glycero-3-phospho-*N*-palmitoylethanolamine, De-NAPE) in mice, as well as the anorectic effect of NAPE (1,2-di-palmitoyl-*sn*-glycero-3-phospho-*N*-palmitoylethanolamine) in NAPE-PLD knockout (NAPE-PLD$^{-/-}$) mice. In both experiments the corresponding PE species, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, was included as a negative control. PA and PC, also in the corresponding species: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine were tested as further controls.

2. Materials and methods

2.1. Chemicals and drugs

1,2-Dihexadecyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, Tween 80, 1,1′-carbonyldiimidazole, and 4-dimethylaminopyridin (DMAP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine was purchased from Larodan AB (Malmö, Sweden). Chloroform, methanol, and acetic acid were obtained from Merck Chemicals (Darmstadt Germany). De-NAPE was synthesized in the laboratory as described later. NAPE and polyethylenglycol were kindly provided by Professor GI Shulman (Yale University) and J Sonnergaard (University of Copenhagen) respectively.

For knockout mice studies; PE and palmitic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). NAPE was synthesized from PE and palmitic acid as described previously [17].

2.2. Animal handling and care

For wild-type animal studies, male C57BL/6 mice of approximately 25 g (Taconic M and B, Ry, Denmark) were maintained at 12:12 h light–dark cycle in temperature and humidity controlled rooms. All animal studies were approved by The Animal Experimentation Inspectorate, The Danish Ministry of Justice. *NAPE-PLD-deficient (NAPE-PLD$^{-/-}$) mice*: NAPE-PLD$^{-/-}$ mice were generated with assistance from Ingenious Targeting Laboratory (Stony Brook, NY). The generation and characterization of the mice will be reported elsewhere by Tsuboi and Ueda. After backcrosses to C57BL/6 genetic background for more than 8 generations, the heterozygotes were intercrossed to give wild-type and NAPE-PLD$^{-/-}$ offspring. The mice were maintained at 12:12 h light–dark cycle (lights on 06:00 am). All animal experiments were approved by Animal Care and Use Committee for Kagawa University.

2.3. Food intake studies in wild-type mice

All mice had free access to standard chow (Altromin #1314, Brogaarden, Gentofte, Denmark) and tap water, and were housed in single cages (TSE LabMaster system). They were randomized for treatment according to their average food intake over a 3-day period of baseline measurements. Prior to the studies, all animals were kept in habituation cages for a minimum of 7 days, adapting them to the feeding system. Before the experiments, animals were sham injected intraperitoneally (*i.p.*) thrice to accustom them to the experimental procedure, twice with saline. Half an hour before lights out, mice were *i.p.* injected with 400 µl vehicle (10% Tween 80/polyethylenglycol 1:1 in saline), 500 mg/kg NAPE, 485 mg/kg De-NAPE, 375 mg/kg PE, 350 mg/kg PA or 395 mg/kg PC. Solutions were prepared by adding Tween 80/polyethylenglycol mixture to PE, De-NAPE or NAPE, stirring, adding saline solution, and sonicating. Food intake, water intake and locomotor activity were measured for 12 h. Metabolic parameters such as oxygen consumption rate (VO2: ml/h/kg), respiratory exchange ratio (RER), and activity (beam breaks) were measured using a sixteen-chamber indirect calorimetry system (TSE Systems, Bad Homburg, Germany). Mice had free access to food and water while in the chambers.

2.4. Food intake studies in NAPE-PLD$^{-/-}$ mice

At 5:30 p.m., ad libitum fed mice were *i.p.* injected with 200 µl of vehicle (10% Tween 80 in saline) or 200 mg/kg body weight of PE or NAPE in the vehicle solution. Solutions were prepared as described earlier. Immediately after the administration, the mice were placed in individual cages, and allowed free access to water, and were fed via feeding equipments with dome-type covers (Roden CAFE, Oriental Yeast Co., Tokyo, Japan). The amount of powder feed consumed was measured at 16 h after the administration.

2.5. Synthesis of De-NAPE

The synthesis of De-NAPE was based on the method of Schmid et al. [17]. In short, a palmitic acid anhydride was formed by reacting free fatty acid with 1,1′carbonyldiimidazole under water free conditions. The anhydride was reacted with 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine, using DMAP as a catalyst, to form De-NAPE. Chloroform was used as solvent. The product was purified by thin