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Enhanced diacylglycerol production by phospholipase D activation is responsible for abnormal increase in concanavalin A cap formation in polymorphonuclear leukocytes from Chediak–Higashi syndrome (beige) mice

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ABSTRACT

We previously reported that enhanced ceramide production induces calpain-mediated proteolysis of protein kinase C (PKC) in leukocytes from Chediak–Higashi syndrome (CHS). In the present study, we demonstrated that phospholipase D (PLD) inhibitors ameliorated abnormal increases in concanavalin A (Con A) cap formation in polymorphonuclear leukocytes (PMNs) from beige mouse, an animal model of CHS. PLD activity in PMNs from beige mice enhanced at 30 to 60 s after Con A stimulation. In Con A-stimulated beige PMNs, both neutral sphingomyelinase (N-SMase) and acidic sphingomyelinase (A-SMase) activities enhanced, and ceramide levels are also increased. We found that ceramide levels were reversed by the treatment of beige PMNs with propranolol which inhibits phosphatidic acid phosphohydrolase. In addition, we showed that diacylgycerol (DAG) analogs enhance both N-SMase and A-SMase activities in PMNs from normal mice. We subsequently examined the association of CHS1 with PLD, and showed that expression of a truncated mutant of *CHS1* in 293T cells induced abnormally rapid activation of PLD after phorbol ester stimulation. Moreover, we showed that specific inhibitors of 14-3-3 proteins, which interact with CHS1/LYST and bind PKC, did not affect abnormal increases in Con A cap formation in beige PMNs. These results suggest that the enhanced DAG production via the PLD pathway is associated with abnormal increases in Con A cap formation in beige PMNs, and that CHS1 may be involved in the regulation of PLD activity.

1. Introduction

Chediak–Higashi syndrome (CHS) is a rare autosomal recessive disorder that is characterized by partial albinism, defective natural killer (NK) activity, reduced bactericidal activity, and increased concanavalin A (Con A) cap formation in polymorphonuclear leukocytes (PMNs) [1,2]. The gene responsible for CHS was identified [3,4] as *CHS1*, which is the human ortholog of the mouse lysosomal trafficking regulator (*Lyst*) gene. Mutations in the *Lyst* gene in mice led to the beige phenotype [5], in which most cells of the body show enlarged lysosome-related organelles [6]. Moreover, specific lysosomal proteases, elastase and cathepsin G, are missorted in CHS and beige cells [7]. The CHS1/LYST protein is thought to play a major role in fission of particular organelle precursors [8]. Mohlig et al. [9] reported that cells lacking a factor associated with neutral sphingomyelinase (N-SMase) activation (FAN) which has structural homology to CHS1, have significantly enlarged lysosomes. Tchernev et al. [10] demonstrated that LYST interacts with four proteins (SNARE-complex proteins, HRS, 14-3-3, and casein kinase 2) important in vesicular transport and signal transduction using yeast two-hybrid screens. It is known that 14-3-3 proteins exist as dimers in cells and function as adaptors that bind to their target proteins in a phosphorylation-dependent manner. 14-3-3 proteins bind protein kinase C (PKC), are phosphorylated by PKC, and inhibit PKC activity [11].

We have previously reported that PKC activity is abnormally down-regulated in PMNs, NK cells, and fibroblasts from beige mice [12–14]. It is known that Con A cap formation is increased in PMNs from beige mice, and we previously reported an important role of PKC in Con A cap formation [15]. We also showed that ceramide, which is recognized

Abbreviations: A-SMase, acidic sphingomyelinase; CHS, Chediak–Higashi syndrome; Con A, concanavalin A; DAG, diacylglycerol; diC8, sn-1,2-dioctanoylglycerol; 2,3-DPG, 2,3-diphosphoglycerate; FAN, factor associated with neutral sphingomyelinase activation; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; NK, natural killer; N-SMase, neutral sphingomyelinase; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PEtOH, phosphatidylethanol; PIP_2, phosphatidyl-inositol 4,5 bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes; PKC, protein kinase C; SM, sphingomyelin; SMase, sphingomyelinase; TLC, thin layer chromatography.

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as an important intracellular second messenger, promotes calpain-mediated downregulation of PKC in murine PMNs and in a neuroblastoma cell line [16,17]. In leukocytes from CHS patients, sphingomyelin (SM) hydrolysis is reportedly accelerated [18]. Furthermore, compared with normal cells, ceramide production by SM hydrolysis is increased in fibroblasts from beige mice [14] and in CHS cell lines, leading to enhanced catalysis of PKC by calpain [19]. This ceramide-induced downregulation of PKC results in abnormal cellular phenotypes, suggesting that abnormal SM metabolic cascades are responsible for the beige phenotype.

Diacylglycerol (DAG) is known to be an acidic sphingomyelinase (A-SMase) activator [20], and can be produced by phosphatidylinositol 4, 5 bisphosphate (PIP_2)-specific phospholipase C (PLC), phosphatidylcholine (PC)-specific PLC, and phospholipase D (PLD) [21]. PLD produces choline and phosphatidic acid (PA), which are then converted to DAG by PA phosphohydrolase [21]. PLD has been recently described as a critical factor for vesicle trafficking events such as exocytosis in neurons [22] and endocrine cells [23]. In mammalian cells, at least two isoforms of PLD exist, PLD1 and PLD2. These isoforms differ in localization and mechanisms of regulation [24].

In the present study, we investigated the factors required for the enhancement of sphingomyelinase (SMase) activities in PMNs from CHS. DAG produced by the abnormal activation of PLD enhanced SMase activities, resulting in increased Con A cap formation in CHS mice. Our data also suggest that CHS1 may play an important role in the regulation of PLD activity.

2. Materials and methods

2.1. Mice

C57BL/6J ($^{+/+}$) and C57BL/6J-beige (bg/bg) mice were originally obtained from CLEA Japan, Inc. (Tokyo, Japan). All mice were housed in a temperature-controlled clean room with a 12-h light/12-h dark cycle. The mice were used in all experiments at 6–10 weeks of age, and all groups were age and sex matched. The mice were anesthetized by inhalation of vaporized halothane. PMNs were obtained by lavage with 5 ml of Hank's balanced salt solution (HBSS) 16–20 h after intraperitoneal injection of 1 ml of sterile thioglycolate broth as described previously [12]. The cells obtained ($5–8 \times 10^6$ cells/mouse) were 80–85% PMNs as determined by May-Giemsa staining. All the animal experiments were approved by the Animal Experiment Committee at the University of Yamanashi.

2.2. Materials

[^{3}H] palmitic acid and [N-methyl-^{14}C] SM were purchased from Moravek Biochemicals (Brea, CA, USA). CAY10593, N-[2-[4-(5-chloro-2, 3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methylethyl]-2-naphthalenecarboxamide), was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Silica gel plates were obtained from Merck (Darmstadt, Germany). R18 peptide and difopein were obtained from Tocris Bioscience (Bristol, UK), and dissolved in dimethylsulfoxide, further diluted with HBSS. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Assays of SMase activity

N-SMase and A-SMase activities were assayed using exogenous [N-methyl-^{14}C] SM by the method of Wiegmann et al. [25]. Murine PMNs (1×10^7/ml) suspended in HBSS were treated with or without Con A (20 μg/ml) for 60 s at 37 °C. When the effects of DAG analogs on SMase activities were examined, cells were treated with HBSS, or DAG analogs for 60 s at 37 °C. To measure N-SMase, cells were washed with HBSS, and dissolved in a buffer containing 20 mM HEPES (pH7.4), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM p-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM phenylmethylsulfonylfluoride, 10 μM leupeptin, 10 μM pepstatin, and 0.2% TritonX-100. After incubation for 5 min at 4 °C, cells were homogenized by repeated squeezing of cells through an 18-gauge needle. Nuclei and cell debris were removed by low speed centrifugation (800 ×*g*). Protein (30 μg) was incubated for 2 h at 37 °C in a buffer (50 μl) containing 20 mM HEPES, 1 mM $MgCl_2$ (pH7.4) and [N-methyl-^{14}C] SM (0.2 μCi/ml). The reaction was linear within this time frame, and the amount of [N-methyl-^{14}C] SM hydrolyzed did not exceed 10% of total amount of radioactive SM added. Phosphorylcholine was then extracted with 800 μl of chloroform/methanol (2:1) and 250 μl of H_2O. Radioactive phosphorylcholine produced from [N-methyl-^{14}C] SM was identified by thin layer chromatography (TLC) and routinely determined in the aqueous phase by scintillation counting. Increment of radioactive phosphorylcholine was confirmed by TLC. To measure A-SMase, cells were dissolved in 200 μl of 0.2% Triton X-100 and incubated for 15 min at 4 °C. Cells were then homogenized and centrifuged (12,000 ×*g*). From the supernatant, protein (30 μg) was incubated for 2 h at 37 °C in a buffer (50 μl) containing 250 mM sodium acetate, 1 mM EDTA (pH5.0) and [N-methyl-^{14}C] SM. Thereafter, the amount of radioactive phosphorylcholine produced was measured as described for N-SMase assay.

2.4. Con A cap formation

Fluorescein isothiocyanate (FITC)-conjugated Con A cap formation was determined as previously described [15,19]. Briefly, PMNs (1×10^7) were suspended in 1 ml of HBSS in silliconized tubes. After cells were incubated with HBSS or various reagents for 30 min at 37 °C, FITC-Con A (20 μg/ml) was added and further incubated for 30 min at 37 °C. The cells were fixed with 2% paraformaldehyde for 10 min, and observed with a fluorescence microscope, BX50 (Olympus, Tokyo, Japan). Scoring was carried out in two categories with respect to the distribution of label as random clusters (diffuse or patched) or capped. Two hundred cells were counted and the percentage of capped cells was calculated.

2.5. Assay of PLD activity in PMNs

PLD activity was assayed according to the method of Schutze et al. [21]. In brief, PMNs were incubated with [^{3}H] palmitic acid (1 μCi/ml) for 1 h at 37 °C in HBSS. The cells were suspended at 10^7 cells/ml in HBSS and incubated with Con A (20 μg/ml) at 37 °C for an indicated period. After cells were washed with HBSS, lipids were extracted as described by Bligh and Dyer [26]. When phosphatidylethanol (PEtOH) was examined, cells were incubated with Con A in the presence of 0.5% ethanol, which leads to the production of PEtOH via PLD-specific transphosphatidylation reaction. Phospholipids were separated using TLC in a solvent system containing chloroform/methanol/acetic acid/water (100:60:20:5). PEtOH was separated from PA and other phospholipids using TLC with a solvent system comprising an organic phase of ethylacetate/acetic acid/water (110:20:110). DAG was separated using TLC with a solvent system of benzene/ethylacetate (6.5:3.5). The individual lipids, identified by co-migration with commercial standards, were visualized in iodine vapor, and the silica gel was scraped to quantify radioactivity. Counts were normalized for total radioactivity in phospholipids to correct for minor differences in labeling between experiments. Incorporation of radioactivity into PC by this method was 35.5 ± 3.6% of total phospholipids.

2.6. Assay for cellular ceramide and SM levels

Cellular ceramide and SM levels were examined according to the method as described previously [14,17]. In brief, PMNs were labeled with [H^3] palmitic acid (1 μCi/ml) for 1 h at 37 °C. After cells were incubated with or without propranolol (200 μM) for 20 min at 37 °C, cells were treated with Con A (20 μg/ml) for 60 s. The cells were precipitated