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Scallop-derived plasmalogens attenuate the activation of PKCδ associated with the brain inflammation

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ABSTRACT

Activation of protein kinase C delta (PKCδ) has been linked to the neuroinflammation but the relationship with the various neurodegenerative diseases including the Alzheimer's disease (AD) was mostly elusive. In the AD brains, the special phospholipids, ethanolamine plasmalogens (Pls), were found to be reduced and our previous study showed that these lipids possess neuroprotective and anti-inflammatory functions. In the present study, we could find that these lipids can significantly attenuate the microglial expression of PKCδ in the neuroinflammation model and in the AD model mice brains. We also show an increase of PKCδ in the human postmortem AD brains. In addition, we also report that scallop derived Pls (sPls) inhibited the p38MAPK and JNK protein expression which are involved in the expressional regulation of PKCδ in the microglial cells. In addition, the lentiviral shRNA-mediated knockdown of PKCδ attenuated the LPS-induced p65 (NF-kB) activation and inflammatory cytokine expression, suggesting that the PKCδ can induce the inflammatory response which can be inhibited by the sPls. Taken together, our recent findings suggest that the sPls can attenuate the increased expression of PKCδ associated with the neuro-inflammation in the murine brain.

1. Introduction

Plasmalogens (Pls) are the unique glycerophospholipids containing a vinyl ether bond at the *sn*-1 position of the glycerol moiety. It was reported that Pls were reduced in the postmortem human Alzheimer's disease (AD) brains [1–3]. In AD people, the hippocampal neurons are greatly damaged resulting in cell death. We have previously reported that the Pls treatments inhibited hippocampal neuronal cell death by increasing the phosphorylation of Akt and ERK proteins [4]. The advanced AD brains are characterized by the progressive loss of neurons due to the intracellular accumulation of toxic proteins such as amyloid-beta peptide (Aβ) and phospho-Tau [5,6]. In addition, the activation of brain glial cells are also involved in the pathologies of AD brains and other age related neurodegenerative diseases [7]. The recent report showed that the oral ingestion of scallop-derived Pls (sPls) significantly improved cognitive function in mild AD patients [8]. In our previous study, we reported that the oral ingestion of Pls inhibited the accumulation of Aβ in the murine brain [9], suggested that oral intake of Pls could improve cognition by inhibiting the AD like pathologies but the mechanism remained elusive.

PKCδ is one isoform of protein kinase C (PKC) family proteins and reported to have many physiological roles related to apoptosis, proliferation and immune responses [10–13]. The PKCδ knockout mice showed a resistance to the inflammatory response [14] and the upregulation of PKCδ in microglial cells promoted neuroinflammation [15]. However, the expressional regulation of PKCδ in the AD pathologies remained mostly elusive. It is known that the LPS binds to TLR4 to activate the caspase-8 and caspase-3, leading to the activation of NF-kB to increase the pro-inflammatory cytokine expression such as TNF-α and IL-1β [15,16]. In regard to the relationship between PKCδ and neurodegenerative diseases, it has been revealed that the Aβ activates PKCδ and the PKCδ expression level is increased in the brain of Parkinson's disease patients [15,17]. However, it is unknown whether the PKCδ is upregulated in the AD brain tissues. We first found that PKCδ is upregulated in the postmortem advanced AD patient's brain tissues. We also found that the reduction of microglial Pls resulted in the upregulation of PKCδ, providing a link with the reduction of Pls in the aged brain.

In the present study, we show for the first time that the sPls have

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the ability to inhibit the induction of pro-inflammatory factor PKCδ *in vitro* and *in vivo*. In addition, the relationship between the induction of PKCδ and reduction of Pls was also confirmed in the cells, suggesting that the Pls might play a very important role in the human brain to inhibit inflammatory signals by suppressing the PKCδ.

2. Materials and methods

2.1. Cell lines and reagents

Human embryonic kidney derived 293FT (HEK293FT) was purchased from Health Science Research Resources Bank, RIKEN, Japan. The BV2 cells were kindly provided by Dr. Hidetoshi Saitoh, Kyushu University, Japan. Cells were maintained by previously described methods [4]. Highly pure sPls were purified from scallop as reported previously [8,18]. The composition of sPls was analyzed by high performance liquid chromatography [18]. sPls are enriched with omega-3 fatty acids, EPA and DHA, compared with the chicken Pls. The fatty acids contents of the Pls derived from scallop (sPls) and chicken breast muscle (Pls) are as follows: DHA (28.7% & 8.2%), EPA (26.1% & 0.0%), arachidonic acid (10.2% & 31.0%), linoleic acid (0.1% and 5.8%), oleic acid/vaccenic acid (1.9% and 20.5%), stearic acid (7.4% and 8.6%), palmitic acid (9.0% and 10.6%) and others (16.7% and 15.4%). sPls were dissolved in 99.5% ethanol to the stock concentration of 10 mg/ml and diluted to the desired concentration (5 μg/ml) immediately before use. Vehicle (ethanol at the same concentration without Pls) was used in the control groups. LPS (Sigma-Aldrich, St. Louis, MO, USA) was used as a pro-inflammatory substance.

2.2. Real time polymerase chain reaction (PCR)

Real time PCR was performed by previously published protocol [19]. The data was analyzed by delta delta Ct technique considering the murine housekeeping gene, glyceraldehyde-3-phosphae dehydrogenase (GAPDH), as an internal control. The sequences of primer pair used in real time PCR were as follows: *GAPDH*, forward 5′-CAATGTGTCCGTCGTGGATCT-3′ and reverse 5′GTCCTCAGTGTAGC CCAAGAT-3'; IL-1β, forward 5′-AAAAAAGCCTCGTGCTGTCG-3′ and reverse 5′-GTCG TTCTTGGTTCTCCTTG-3'; TNF-α, forward 5′-AAG CCTGTAGCCCACGTCGT-3′ and reverse 5′-AGGTACAACCCATCGGC TGG-3'.

2.3. Western blot analysis

The western blotting (WB) experiments were carried out by following the previously described protocol [20]. Mouse PKC delta, rabbit p38MAPK, rabbit JNK, and mouse p65 antibodies were purchased from cell signaling technology. We also used rabbit GNPAT (Abcam), mouse anti-β-Actin (Santa Cruz), and mouse anti-Lamin (BD biosciences). Quantification of the WB images were performed by Image-J software as described before [4,19].

2.4. Preparation of sh-RNA lentiviruses and cell transfection

The sh-RNA lentiviruses were prepared by following the previously published protocol [20]. The target sequences were as follows: sh-Luc (Luciferase): (5′-CTTACGCTGAGTACTTCGAG-3′), sh-PKCδ: (5′-TTGCAAACAGTCTATGCG-3′) and sh-GNPAT: (5′-GTCCCAATTAGCATCAGT-3′). The lentivirus particles (2×10^5 transduction unit) were subjected to the 6 cm dish of BV2 microglial cells for 48 h to knockdown the genes.

2.5. Immunocytochemical (ICC) and immunohistochemical (IHC) assays

The ICC and IHC studies were performed according to the protocols of our previous published papers [20]. The primary antibodies

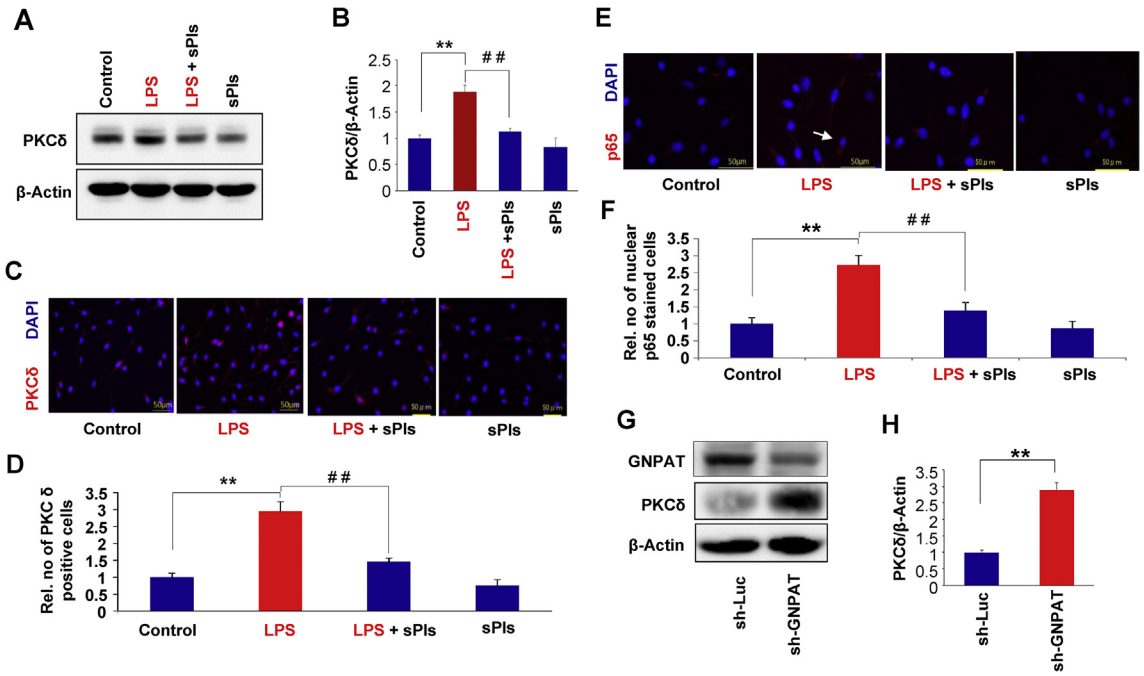

Fig. 1. SPls attenuated PKCδ and NF-kB activation upon inflammatory stimulus.
(**A-B**) Western blotting and its quantification data showed that the LPS (1 μg/ml) treatments for 6 h induced PKCδ expression in the BV2 microglial cells and it was attenuated by pretreatments with the sPls (5 μg/ml) for 12 h ($^{**}P < 0.01$ against the control, $^{\#\#}P < 0.01$ against the LPS group, Bonferroni's tests, n = 5). (**C-F**) In the same experimental condition of panel **A**, the ICC data showed the relative expression of PKCδ stained microglial cells (C and D) and the nuclear p65 stained cells (E and F). The data represents mean ± SEM ($^{**}P < 0.01$ against the control, $^{\#\#}P < 0.01$ against the LPS group, Bonferroni's tests, n = 5). The scale bars indicate 50 μM. (**G-H**) Western blotting data showed that the reduction of Pls in BV2 microglial cells by knockdown of sh-GNPAT lentivirus induced the protein expression of PKCδ ($^{**}P < 0.01$, n = 5).

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