Unexpected role of lipocalin-type prostaglandin D synthase in brain
Regulation of glial cell migration and morphology

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Lipocalin-type prostaglandin D synthase (L-PGDS) is one of the most abundant proteins in the cerebrospinal fluid. Nevertheless, its role in the central nervous system is far from clear. Here, we present evidence that L-PGDS induces glial cell migration and morphological changes in vitro and in vivo. We also identified myristoylated alanine-rich C-kinase substrate (MARCKS), heat shock proteins and actin as L-PGDS-binding proteins, demonstrating that MARCKS/Akt/Rho/Jnk pathways are involved in the L-PGDS actions in glia. We further show that the cell migration-promoting activity of L-PGDS is independent of PGD2 production. The results suggest a novel non-enzymatic function of L-PGDS protein in brain inflammation, and may have an impact on glial cell biology and brain pathology related with reactive gliosis. L-PGDS is a potential drug target that can be exploited for therapeutic intervention of glia-driven neuroinflammation and related diseases.

Keywords: glia, cell migration, morphology, L-PGDS, MARCKS, neuroinflammation, cerebrospinal fluid

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L-PGDS protein present in cerebrospinal fluid or locally secreted in the inflammatory site may modulate glial recruitment into the injury site in the CNS. In an attempt to elucidate the molecular mechanisms underlying the L-PGDS-induced glial cell migration, L-PGDS-interacting proteins were identified by coimmunoprecipitation followed by liquid
Chromatography and tandem mass spectrometry (LC-MS/MS) analysis. In order to identify the L-PGDS-binding proteins on or near the cell surface, intact glial cells were treated with L-PGDS protein, thoroughly washed prior to formaldehyde-mediated crosslinking. L-PGDS-treated glial cells were then lysed and immunoprecipitated using the anti-L-PGDS antibody. The proteins coimmunoprecipitated with L-PGDS were separated by PAGE and visualized by silver staining, which were then identified with LC-MS/MS analysis. MARCKS, actin and a group of heat shock proteins were identified as the major L-PGDS-binding proteins (Table 1). The interaction between L-PGDS and MARCKS was confirmed by a separate immunoprecipitation and western blot analysis. An important role of MARCKS in the L-PGDS-induced glial migration was demonstrated by knocking down MARCKS expression using siRNA. MARCKS knockdown partially abrogated

Table 1. List of proteins coimmunoprecipitated with L-PGDS

| Accession number* | Protein name                      | Symbol  | Peptide hit** |
|-------------------|-----------------------------------|---------|---------------|
| IPI00554929       | Heat shock protein HSP 90-β       | Hsp90ab1| 31            |
| IPI00110850       | Actin, cytoplasmic 1              | Actb    | 25            |
| IPI00223357       | Heat shock cognate 71 kDa protein | Hspa8   | 12            |
| IPI00319992       | 78 kDa glucose-regulated protein  | Hspa5   | 7             |
| IPI00462072       | α-enolase                         | Eno1    | 7             |
| IPI00129526       | Hsp90b1 endoplasm                 | Hsp90b1 | 5             |
| IPI00275539       | Reticulon 4                       | Rtn4    | 4             |
| IPI00229534       | Myristoylated alanine-rich C-kinase substrate | Marcks | 4             |
| IPI00111560       | Isoform 1 of protein SET          | Set     | 3             |
| IPI00115679       | Isoform 2 of neutral α-glucosidase AB | Ganab | 3             |
| IPI00133903       | Stress-70 protein, mitochondrial  | Hsp9a9  | 2             |
| IPI00604969       | Titin isoform N2-A                | Tnt     | 2             |

*SWISS-PROT accession numbers are listed. **Number of peptide hit identified by LC-MS/MS analysis. In brief, NIH3T3 fibroblast cells were treated with the recombinant L-PGDS protein (1 μg/ml), and then crosslinked with 1% formaldehyde for 1 h and rinsed twice with PBS. Cells were lysed in triple-detergent lysis buffer (50 mM TRIS-HCl, pH 8.0; 150 mM NaCl; 0.02% sodium azide; 0.1% SDS; 1% Nonidet P-40; 0.5% sodium deoxycholate; and 1 mM phenylmethyl sulfonyl fluoride). The lysates were centrifuged for 20 min at 4°C, and the supernatants were collected. The protein concentration in the cell lysates was determined using the Quant-iT Protein Assay kit (Molecular Probes). To remove nonspecific binding proteins in the lysates, the samples were incubated in a ~30 μl packed volume of recombinant protein G-agarose (PGA) for 1 h at 4°C. After a brief centrifugation, supernatants were collected and then incubated with anti-L-PGDS antibody (1 μg/ml; Cayman Chemical) for 4 h at 4°C. PGA (30 μl) was then added and incubated for 4 h. Afterwards, L-PGDS-Ab-PGA complexes were washed three times with wash buffer (50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol). For the identification of coimmunoprecipitated proteins, the immunoprecipitation samples were separated by electrophoresis on a 10% polyacrylamide gel and visualized by silver staining. The protein band of interest was excised from the silver-stained gel for in-gel tryptic digestion. The excised gel slices were destained and shrunk by dehydration in acetonitrile and dried in a vacuum centrifuge. Proteins within the shrunk gel slices were then digested overnight with trypsin at a substrate/enzyme ratio of 10:1 (wt/wt) in 25 mM ammonium bicarbonate (pH 8.0). The enzyme reaction was terminated by the addition of 0.1% formic acid in water. Peptides from gel pieces were extracted by sonication for 10 min and supernatants containing the peptides were transferred to new tubes. Peptides were analyzed using a liquid chromatography (LC) and tandem mass spectrometry (MS/MS) system with reverse-phase LC, which consisted of a Surveyor MS pump (Thermo Electron), a Spark autosampler (Spark Holland), and a Finnigan LTQ linear ion-trap mass spectrometer (Thermo Electron) equipped with nanospray ionization sources. All MS/MS data were searched against the IPI mouse protein database (version 3.16) using the SEQUEST algorithm (Thermo Electron) incorporated into BioWorks software (version 3.2).
the L-PGDS effects on glial cell migration. Further studies using pharmacological inhibitors, dissection of intracellular signal transduction pathways, and morphological analysis revealed that L-PGDS induced glial cell migration via MARCKS/Akt/Rho/Jnk pathways, leading to augmented formation of actin filaments and focal adhesion (Fig. 1). The list of proteins co-immunoprecipitated with L-PGDS also included heat shock proteins, actin, α-enolase and reticulon-4. However, mechanistic involvement of these proteins in the L-PGDS actions in glia remains to be determined. Heat shock proteins and actin may indirectly interact with L-PGDS through membrane-anchoring adaptor proteins (Fig. 1), suggesting that stress response and actin cytoskeleton may be associated with L-PGDS effects on glial cell morphology and motility. α-enolase is a glycolytic enzyme that is expressed in most tissues.14 α-enolase has been identified as an autoantigen in Hashimoto encephalopathy, asthma and Behcet disease. It is also known as the Myc-binding protein-1 (MBP1), which regulates c-myc activity. The interaction between L-PGDS and α-enolase has to be confirmed and deserves further investigation. Reticulon-4, also known as Nogo, is an inhibitor of neurite outgrowth in CNS.15 Reticulon-4 is associated with endoplasmic reticulum, and has a potent inhibitory effect on neurite outgrowth, which blocks the CNS regeneration. Membrane-associated reticulon-4 binds to its receptor (NgR) to inhibit axon outgrowth. Blockade of reticulon-4 during neuronal damage is thought to enhance restoration of damaged neurons. As the neurite outgrowth requires local movement of cellular processes, interaction between L-PGDS and reticulon-4 may regulate axonal outgrowth. The precise role of reticulon-4 in the L-PGDS actions may need to be clarified by further investigation.

Although much of the findings in the study by Lee et al. were based on in vitro experiments, the effect of L-PGDS on glial migration was also determined in vivo. When L-PGDS protein was stereotaxically injected into the specific regions of the mouse brain such as striatum or cortex, the number of GFAP-positive astrocytes in the peri-region of the L-PGDS-injected site was significantly higher compared with the vehicle-injection (Fig. 2). The results are based on immunofluorescence detection of GFAP-positive astrocytes from the six independent tissue sections per animal. Astrocyte count obtained from three different animals per group (vehicle vs. L-PGDS-injected mice) showed statistically significant differences. These results support that L-PGDS enhances glial migration and accumulation in brain. These phenotypic changes of glia are well known to be associated with reactive gliosis. Therefore, the recent report by Lee et al. suggests a novel non-enzymatic role of L-PGDS in brain inflammation, and has an impact on glial cell biology and brain pathology that is related with reactive gliosis. Since cell migration plays a pivotal role in development, wound healing, immune/inflammatory responses and tumor metastasis, the effects of L-PGDS on cell migration and morphology identified in the study by Lee et al. may broaden our understanding of these biological processes. In conclusion, L-PGDS is a potential drug target that can be exploited for therapeutic intervention of glia-driven neuroinflammation and related diseases.
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