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Glutamine up-regulates MAPK phosphatase-1 induction via activation of Ca^{2+} → ERK cascade pathway

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ABSTRACT

The non-essential amino acid L-glutamine (Gln) displays potent anti-inflammatory activity by deactivating p38 mitogen activating protein kinase and cytosolic phospholipase A_2 via induction of MAPK phosphatase-1 (MKP-1) in an extracellular signal-regulated kinase (ERK)-dependent way. In this study, the mechanism of Gln-mediated ERK-dependency in MKP-1 induction was investigated. Gln increased ERK phosphorylation and activity, and phosphorylations of Ras, c-Raf, and MEK, located in the upstream pathway of ERK, in response to lipopolysaccharide*in vitro* and *in vivo*. Gln-induced dose-dependent transient increases in intracellular calcium ($[Ca^{2+}]_i$) in MHS macrophage cells. Ionomycin increased $[Ca^{2+}]_i$ and activation of Ras → ERK pathway, and MKP-1 induction, in the presence, but not in the absence, of LPS. The Gln-induced pathways involving Ca^{2+} → MKP-1 induction were abrogated by a calcium blocker. Besides Gln, other amino acids including L-phenylalanine and L-cysteine (Cys) also induced Ca^{2+} response, activation of Ras → ERK, and MKP-1 induction, albeit to a lesser degree. Gln and Cys were comparable in suppression against 2, 4-dinitrofluorobenzene-induced contact dermatitis. Gln-mediated, but not Cys-mediated, suppression was abolished by MKP-1 small interfering RNA. These data indicate that Gln induces MKP-1 by activating Ca^{2+} → ERK pathway, which plays a key role in suppression of inflammatory reactions.

1. Introduction

The non-essential amino acid L-Glutamine (Gln) is an energy substrate for most cells [1,2], and is important in multiple ways in the nitrogen- and carbon-skeleton exchange among different tissues [3]. Several studies have demonstrated that Gln has an anti-inflammatory activity in humans [4] and animals [5,6].

Regarding the molecular mechanism of anti- inflammatory activity of Gln, we have shown that Gln was beneficial against endotoxin shock as well as bronchial allergic asthma by inhibiting phosphorylation and activity of cytoplasmic phospholipase A_2 ($cPLA_2$) [7,8], which has a high selectivity for liberating arachidonic acid that is subsequently metabolized by a panel of downstream enzymes for eicosanoid production [9,10]. We have subsequently demonstrated that Gln deactivates p38 and c-Jun N-terminal kinase(JNK) mitogen-activated protein kinases (MAPKs) by a rapid induction of MAPK phosphatase 1 (MKP-1) protein in an extracellular signal-regulated kinase (ERK)-dependent way, which was beneficial against fatal endotoxic shock [11], steroid-resistant airway neutrophilia in allergic asthma [12] and, in particular, dermatitis [13,14] in mice.

MKP-1, a member of the MKP family, plays a pivotal role in the negative control of p38 and JNK [15,16]. Given that p38 has a role in the production of inflammatory molecules [17,18], MKP-1 has been known to functions as a critical negative regulator of inflammation. MKP-1 is a labile protein that is normally degraded via the ubiquitin/proteasome pathway, and its phosphorylation

Abbreviations: AP-1, activating protein 1; Ala, alanine; Asp, aspartate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethylester; CaM, calmodulin; CaR, Ca^{2+}-sensing receptor; CD, contact dermatitis; $cPLA_2$, cytoplasmic phospholipase A_2; DMSO, dimethyl sulfoxide; DNFB, 1-fluoro-2,4-dinitrobenzene; ESR, ear swelling response; ERK, extracellular signal-regulated kinase; Gln, L-glutamine; Glu, glutamate; Gly, glycine; H&E, hematoxylin and eosin; $[Ca^{2+}]_i$, intracellular calcium concentration; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen activated protein kinase; MKP-1, MAPK phosphatase-1; PEI, polyethyleneimine; siRNA, small interfering RNA

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reduces its ubiquitination and degradation [19–21]. ERK MAPK phosphorylates MKP-1 on two carboxyl-terminal serine residues -serine 359 and serine 364, which stabilizes MKP-1 by preventing the degradation from ubiquitin/proteasome pathway [19]. We have also demonstrated that ERK inhibitors blocked Gln-induced MKP-1 phosphorylation and protein induction [11–13], further supporting a role for ERK in Gln induction of MKP-1.

In this study, we investigated the precise mechanism of Gln-mediated MKP-1 induction. We found that Gln upregulates MKP-1 expression by activating initial Ca^{2+} response, followed by Ras/c-Raf/MEK/ERK pathway.

2. Materials and methods

2.1. Animals

Female BALB/c mice (7–8 weeks old, 16–18 g body weight) were purchased from the Samtako Bio Korea, and kept in our animal facility for at least 1 week before use. All animals used in this study were handled using the protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

2.2. Chemicals and reagents

DNFB, LPS derived from *Escherichia coli* O127:B8 (L3024), and all L-amino acids used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). U0126, a specific inhibitor of MEK1/2, was obtained from Calbiochem (Madison, WI, USA). U0126 dissolved in DMSO (12.5 mg/kg) [11]was injected i.p. 24 h before LPS treatment. The control group received vehicle. The intracellular calcium chelator, BAPTA-AM was purchased from Calbiochem (Madison, WI, USA). Fluo 4/AM were purchased from Molecular Probes (Eugene, OR, USA). Primary antibodies (rabbit *anti*-Ras, *anti*-phospho-c-Raf (Ser^{338}), anti-phospho-MEK1/2 ($Ser^{217/221}$), *anti*-phospho-ERK1/2, and *anti*-phospho-MKP-1) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-MKP-1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and *anti*-phospho-MBP (clone P12) was from Millipore Corporation (Billerica, MA, USA).

2.3. Cell culture

MHS murine alveolar macrophage cells (ATCC CRL-2019), were maintained in RPMI 1640 supplemented with 10% heat inactivated FBS (Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere.

2.4. Immunoblotting

Mice were sacrificed by cervical dislocation. Ear samples were frozen in liquid nitrogen and were stored in −70 °C until analyzed. Ear samples were homogenized in the Phosphosafe Extraction Reagent (Novagen, Madison, WI). Immunoblotting analysis was performed as described previously[13].

2.5. Immunoprecipitation

Lungs and cells were lysed in non-denaturing lysis buffer containing 20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol, and 1% Triton X-100, 2 mM EDTA, protease inhibitor cocktail, and phosphatase inhibitor. Equal amounts of cell or tissue extracts were incubated with *anti*-phospho ERK1/2 at a dilution of 1:50 for 4 h at 4 °C in the same total volume of lysis buffer thereafter, protein A/G conjugated agarose beads (Santa Cruz Biotechnology) was added and incubated overnight. The agarose beads containing the immunoprecipitate was then washed with the lysis buffer five times and finally collected by centrifugation. After keeping a small amount of the beads for the kinase assay, the rest of the beads were suspended in sample buffer and boiled for Western blot analysis.

2.6. Assay of ERK activity

After challenge, lung samples were weighed (100 mg) and homogenized in 1 ml of in non-denaturing lysis buffer containing 20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol, and 1% Triton X-100, 2 mM EDTA, protease inhibitor cocktail, and phosphatase inhibitor. Homogenates were then centrifuged at 12,000 *g* for 20 min at 4 °C to obtain the supernatant. Equal amounts of cell or tissue extracts were incubated with anti- ERK1/2 at a dilution of 1:50 for 4 h at 4 °C in the same total volume of lysis buffer thereafter, protein A/G conjugated agarose beads (Santa Cruz Biotechnology, CA, USA) was added and incubated overnight. The agarose beads containing the immunoprecipitate was then washed with the lysis buffer five times and finally collected by centrifugation. The washed precipitate was resuspended in 30 μl kinase buffer (15 mM Tris/HCl, pH 7.2, 15 mM $MgCl_2$, and 1 mM dithiothreitol). ERK activity assay was analyzed using MAP kinase assay kits (Merckmilipore, Darmstadt, Germany) according to the manufacturer's instructions. The assay is based on the ability of ERK to phosphorylate the specific substrate, myelin basic protein, (MBP). The phosphorylated MBP is then analyzed by immunoblot analysis, probing with a monoclonal Phospho-specific MBP antibody.

2.7. Assay of Ras activation

Ras activation was evaluated by measuring an increase in intracellular Ras protein levels as described elsewhere [22,23].

2.8. Measurement of $[Ca^{2+}]_I$

MHS cells were washed with Hanks' balanced salt solution (HBSS; 2 mM $CaCl_2$, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 20 mM HEPES, pH 7.3) containing 1% bovine serum albumin (BSA). MHS cells were incubated with 5 μM Fluo 4/AM (Molecular Probes) in Hanks' balanced salt solution for 45 min at 37 °C. The cells were washed three times with HBSS. The MHS cells were placed on the stage of confocal microscope (Nikon, Tokyo, Japan) and Fluo 4/AM loaded cells were excited at excitation wavelength (*F*488 nM) and an emission fluorescence was measured at 530 nm. For the calculation of $[Ca^{2+}]_i$, the method of by Tsien et al.[24]was used with the following equation: $[Ca^{2+}]_i = Kd(F\text{-}F_{min})/(F_{max}\text{-}F)$, where *K*dis 345 nM for Fluo-4, respectively, and *F* is the observed fluorescence levels. Each tracing was calibrated for the maximal intensity (F_{max}) by the addition of ionomycin (10 μM) and for the minimal intensity (F_{min}) by the addition of EGTA (50 mM) at the end of each measurement. The specific inhibitor for intracellular Ca^{2+} chelator (BAPTA-AM) was incubated at a suboptimal concentrations of 50 μM. The inhibitor was diluted into DMSO. To study Ca^{2+} entry in cells, Ca^{2+} free conditions were used.

2.9. Induction of CD

Induction of CD was performed as described previously [13].