Glutamine up-regulates MAPK phosphatase-1 induction via activation of $Ca^{2+} \rightarrow$ 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 $\rightarrow$ ERK pathway, and MKP-1 induction, in the presence, but not in the absence, of LPS. The Gln-induced pathways involving $Ca^{2+} \rightarrow$ 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 $\rightarrow$ 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 $Ca^{2+} \rightarrow$ 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

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 (F488 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: $[Ca^{2+}]_i = K_d(F\text{-}F_{min})/(F_{max}\text{-}F)$, where K_d is 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].

2.10. Measurement of ESR

ESR was measured as described previously [13].

2.11. Histological analysis

Histological analysis was performed as described previously [13].

2.12. Measurement of cytokines and LTB$_4$

Supernatants from ear samples were prepared as described previously [13]. Levels of IFN-γ, IL-1β and TNF-α were analyzed by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. ELISA kit for LTB$_4$ was from Cayman Chemical Company (Ann Arbor, MI, USA).

2.13. siRNA interference

siRNA strands for mouse MKP-1 and controls were obtained from Santa Cruz Biotechnology. *In vivo* delivery of siRNA was performed using *in vivo*-jet polyethyleneimine (PEI, Polyplus-transfection) (BP 90,018, F-67401 ILLKIRCH CEDEX, France), according to the manufacturer's instructions. To confirm that the MKP-1 siRNA used really blocked the synthesis of its target, an

immunoblotting analysis was performed.

2.14. Statistical analyses

All data are shown as mean $\pm$ error of the mean ($\pm$ SEM). Statistical comparison was performed using one-way ANOVA followed by the Fisher test. Significant differences between the groups were determined using the unpaired Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Gln increases ERK phosphorylation and activity

Systemic administration of Gln 5 min after exposure to lipopolysaccharide (LPS) resulted in appearance of MKP-1 phosphorylation and protein as early as 5 min in the lungs (Fig. 1(A)). The effects were abrogated by the MEK inhibitor, U0126 (Fig. 1(B)), confirming our previous findings that Gln induction of MKP-1 is ERK-dependent [11–13]. To investigate the underlying mechanism of ERK dependency, we assessed the possibility that Gln could affect ERK activity. Gln was administered i.p. at 5 min post-LPS, and ERK phosphorylation and activity were observed at 10, 15, and 30 min post-LPS. Gln increased ERK phosphorylation, which was
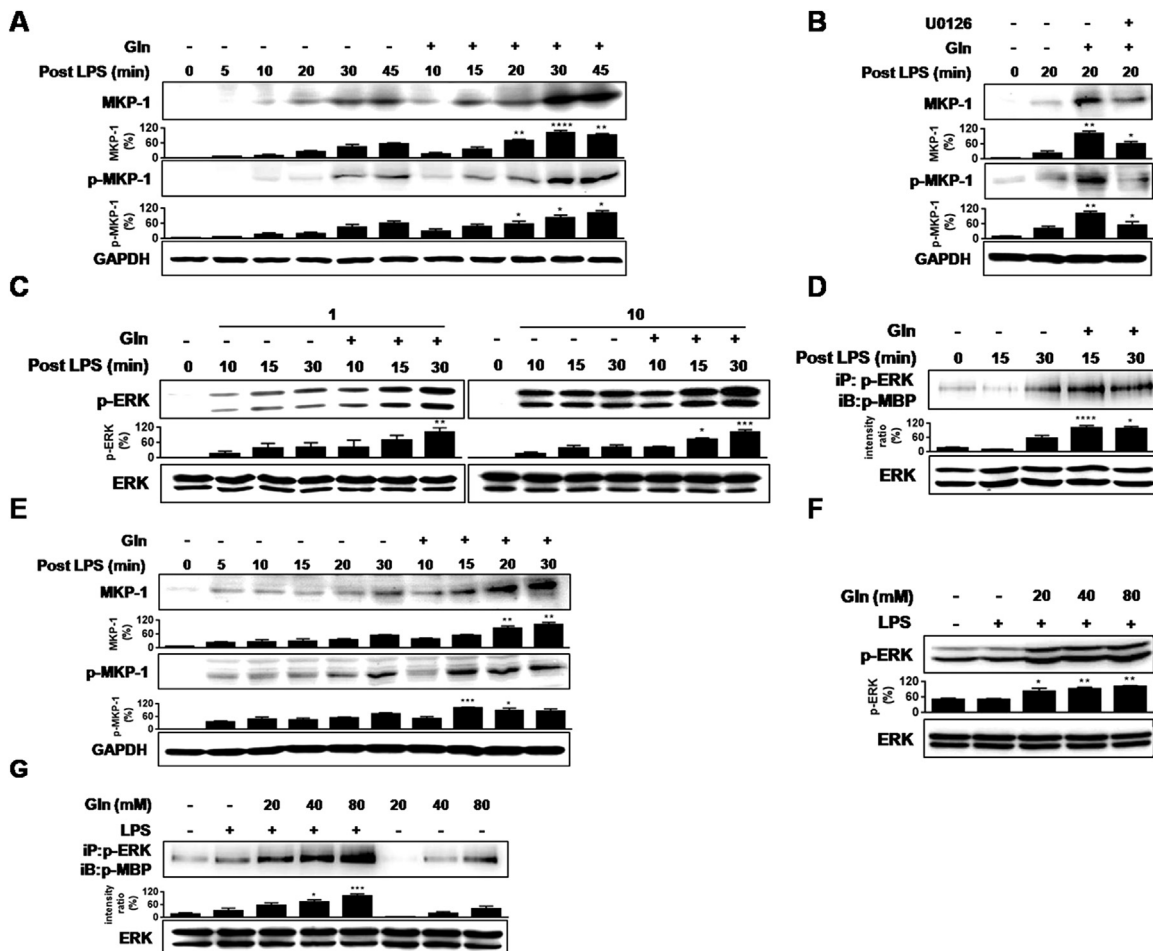

Fig. 1. Gln induces MKP-1 induction in an ERK-dependent way. (A) Lungs were removed at the indicated times after i.v. injection of LPS (50 µg/kg). Gln (750 mg/kg) was given i.p. 5 min after LPS. (B) U0126 (12.5 mg/kg) was injected i.p. 24 h before LPS injection. Lungs were removed at 20 min (C) Gln (750 mg/kg) was given i.p. 5 min after LPS (1 µg or 10 µg/mouse). (D) ERK activity against MBP was performed as described in Materials and Methods. A representative of three to five independent experiments with 3–5 mice/time point/experiment is shown.(E). Gln (40 mM) was added to MHS cells (2×10^6) 5 min after LPS (1 ng/ml) stimulation. (F) and (G). Gln (20 mM, 40 mM or 80 mM) was added to MHS cells (2×10^6) 5 min after LPS (1 ng/ml) stimulation. Cells were stimulated for 15 min A representative of three independent experiments is shown.
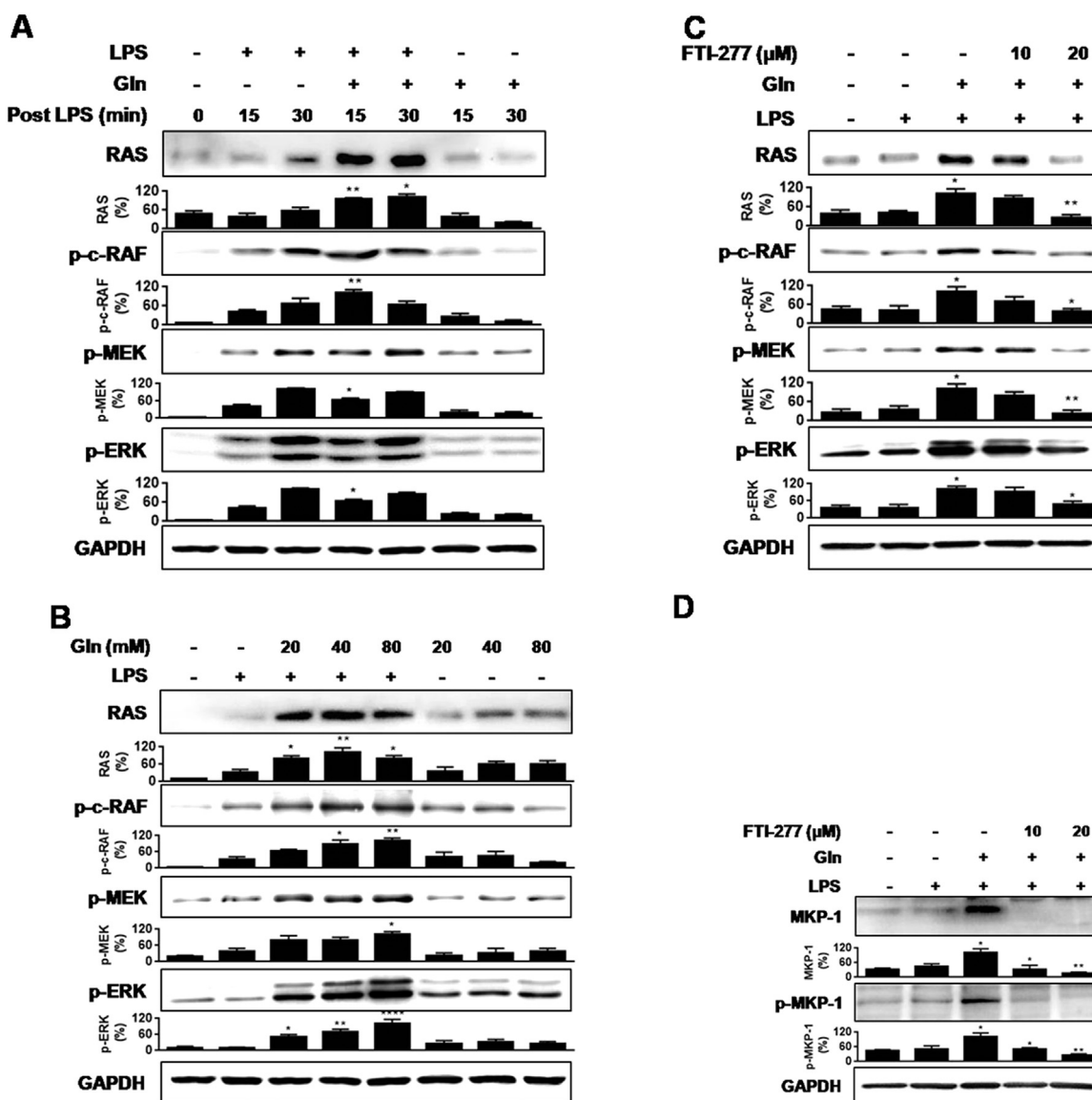
Fig. 2. Gln activates Ras/c-Raf (Ser[338])/MEK/ERK pathway in response to LPS. (A) Lungs were removed at the indicated times after i.v. injection of LPS (50 μg/kg). Gln (750 mg/kg) was given i.p. 5 min after LPS. A representative of five independent experiments with 3–5 mice/time point/experiment is shown. (B) Gln (40 mM) was added to MHS cells (2×10^6) 5 min after LPS (1 ng/ml) stimulation (C) and (D) Serum starved MHS cells were pretreated with FTI (10 μM or 20 μM) for 1 h before stimulation with LPS (1 ng/ml). Gln (40 mM) was added to MHS cells 5 min after LPS. Cells were stimulated for 15 min A representative of three to five independent experiments is shown.

more prominent at the relatively lower concentrations of LPS (1 μg/mouse) than at a higher concentration (10 μg/mouse) (Fig. 1 (C)). Gln also increased ERK activity with a similar LPS concentration kinetics, as was the case for ERK phosphorylation (Fig. 1 (D)). We also examined the effect of Gln in the murine alveolar macrophage cell line (MHS). LPS-induced MKP-1 phosphorylation and protein induction at around 30 min, and addition of Gln at 5 min after LPS stimulation resulted in early phosphorylation and protein induction of MKP-1 as early as within 5 min (Fig. 1(E)). Additionally, Gln increased not only ERK phosphorylation (Fig. 1 (F)), but also ERK activity (Fig. 1(G)) in dose-dependent manners, as was evident *in vivo*.

3.2. Gln activates Ras/c-Raf/MEK/ERK pathway in response to LPS

Regarding the upstream pathway of ERK activation, the Ras/Raf/MEK/ERK pathway (ERK cascade) is well established [25–28]. Therefore, we assessed whether Gln can activate the pathway. Gln increased the levels Ras protein, and phosphorylations of c-Raf

(Gln failed to phosphorylate b-Raf, data not shown) and MEK *in vivo* (Fig. 2(A)) as well as *in vitro* (Fig. 2(B)). Gln did not exert such effects in the absence of LPS especially *in vivo*. The Ras inhibitor, farnesyl transferase inhibitor (FTI)-277, inhibited Gln-mediated increases of Ras, phosphorylation of c-Raf, MEK, and ERK (Fig. 2(C)), as well as MKP-1 phosphorylation and protein induction (Fig. 2(D)) in MHS cells. The data indicate that Gln induction of MKP-1 is mediated via activation of ERK cascade.

3.3. Gln activates ERK cascade by increasing intracellular Ca^{2+} level

How can Gln modulate the Ras signal? Given that L-amino acids have been reported to increase intracellular calcium ($[Ca^{2+}]_i$) level [29–31]and the positive modulation of Ras signaling pathways by an increase in $[Ca^{2+}]_i$ [32–35], we investigated the possible involvement of Ca^{2+} in MHS cells. LPS-induced a transient rise in $[Ca^{2+}]_i$, and addition of Gln 3 min after LPS-induced another strong Ca^{2+} increase (Fig. 3(A)). In the absence of LPS, Gln also induced a transient rise in $[Ca^{2+}]_i$ (Fig. 3(B)). In both cases,
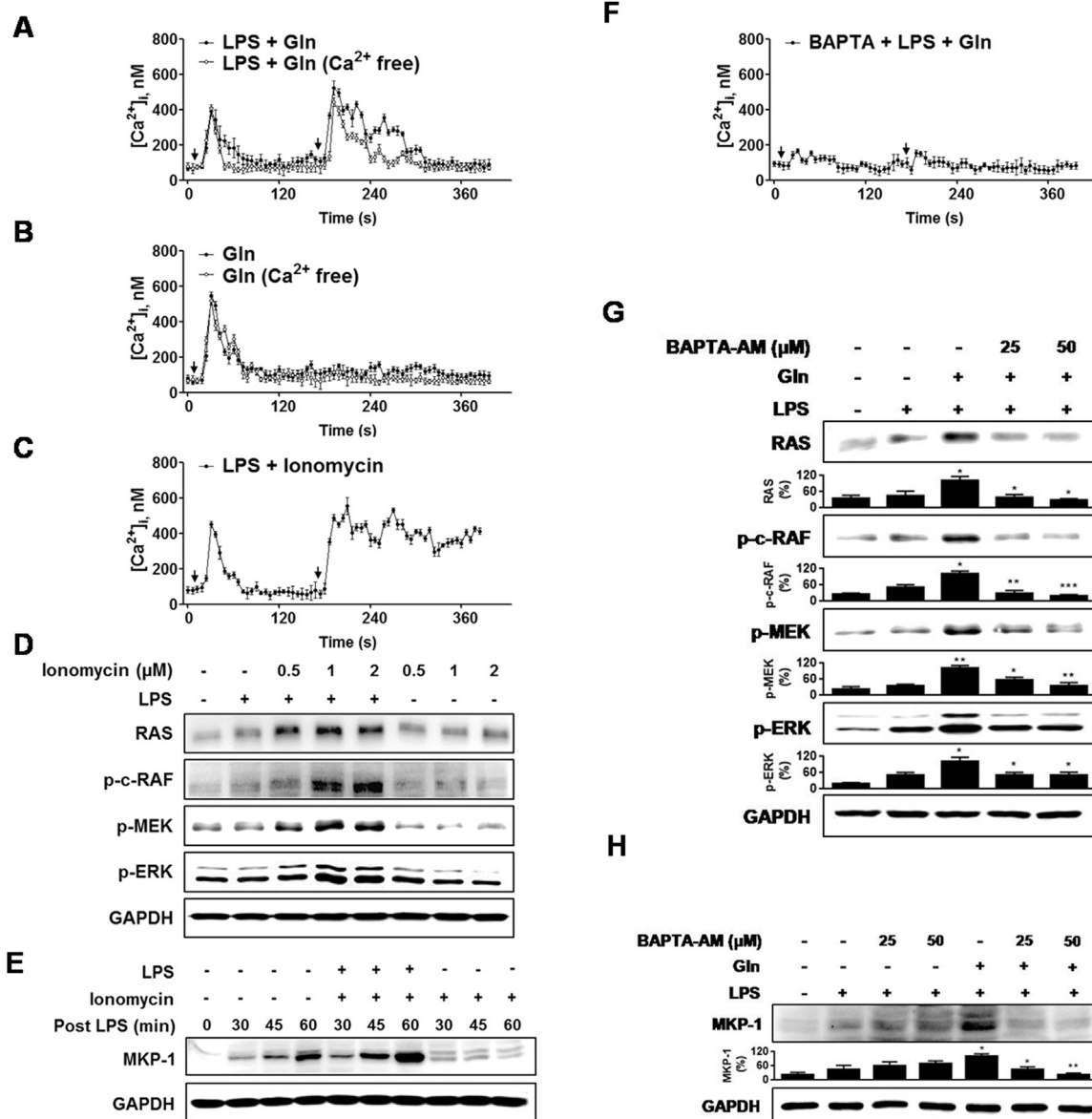
Fig. 3. Gln activates ERK cascade via increasing $[Ca^{2+}]_i$. (A) MHS cells (2×10^6) were loaded with Fluo 4/AM and then stimulated with the LPS (1 ng/ml) and then with Gln (40 mM) 3 min later. (B) $[Ca^{2+}]_i$ response in the absence of LPS. (C) Ionomycin (1 μM), 3 min after LPS. (D) Five min after LPS, cells were stimulated with Ionomycin for 15 min (E) Ionomycin, 5 min after LPS. (F) Cells were pretreated with BAPTA-AM (50 μM) for 30 min before LPS. (G) and (H) Serum starved cells were pretreated with BAPTA-AM for 30 min before LPS and then Gln 5 min later. Cells were cultured for 15 min A representative of three to five independent experiments is shown. Each point represents mean $\pm$ S. E. of $[Ca^{2+}]_i$(A–C and F).

Gln-mediated elevation in $[Ca^{2+}]_I$ was not significantly affected by removal of extracellular Ca^{2+}.

We next examined whether an increase in $[Ca^{2+}]_i$ is responsible for the Gln-induced activation of the pathways involving Ras → ERK → MKP-1 using ionomycin. Ionomycin increased the $[Ca^{2+}]_i$ level in the presence of LPS (Fig. 3(c)) and increased phosphorylation of Ras and ERK (Fig. 3(D)) and induction of MKP-1 (Fig. 3(E)), in the presence, but not in the absence, of LPS. Furthermore, intracellular calcium chelator, BAPTA-AM, abrogated Gln-induced Ca^{2+} response (Fig. 3(F)), phosphorylation of Ras, c-Raf, MEK, and ERK (Fig. 3(G)), and MKP-1 induction (Fig. 3(H)). Taken together, the data indicate that Gln induction of MKP-1 is attributed to its ability to induce a rise in $[Ca^{2+}]_i$, which in turn activates ERK cascade.

3.4. Comparison of other amino acids in their abilities to activate

Ca^{2+}/Ras/ERK/MKP-1 pathway

We further investigated the abilities of other amino acids to activate the pathways, Ca^{2+}/Ras/ERK/MKP-1. Besides Gln, we included the following seven L-amino acids according to their differences in R groups: Glycine (Gly) and alanine (Ala) (nonpolar, aliphatic), phenylalanine (Phe) and tryptophan (Trp) (aromatic), lysine (Lys) and arginine (Arg) (positively charged), and cysteine (Cys) (polar, uncharged). Gln has a polar, uncharged R group like Cys. We excluded the negatively charged group, aspartate and glutamate because of their poor solubility. In the presence of LPS, only Gln, Cys, and Phe-induced a transient Ca^{2+} increase (Fig. 4 (A)) in MHS cells, in the order of their effectiveness: Gln > Cys > Phe. In the presence of LPS, Gln, Cys, Phe, and Trp were capable of mobilizing Ca^{2+}, in the order of their effectiveness: Gln > Cys > Phe > Trp (Fig. 4(B)). Other amino acids were inactive. Taken together, these data indicated that the polar, uncharged group (Gln
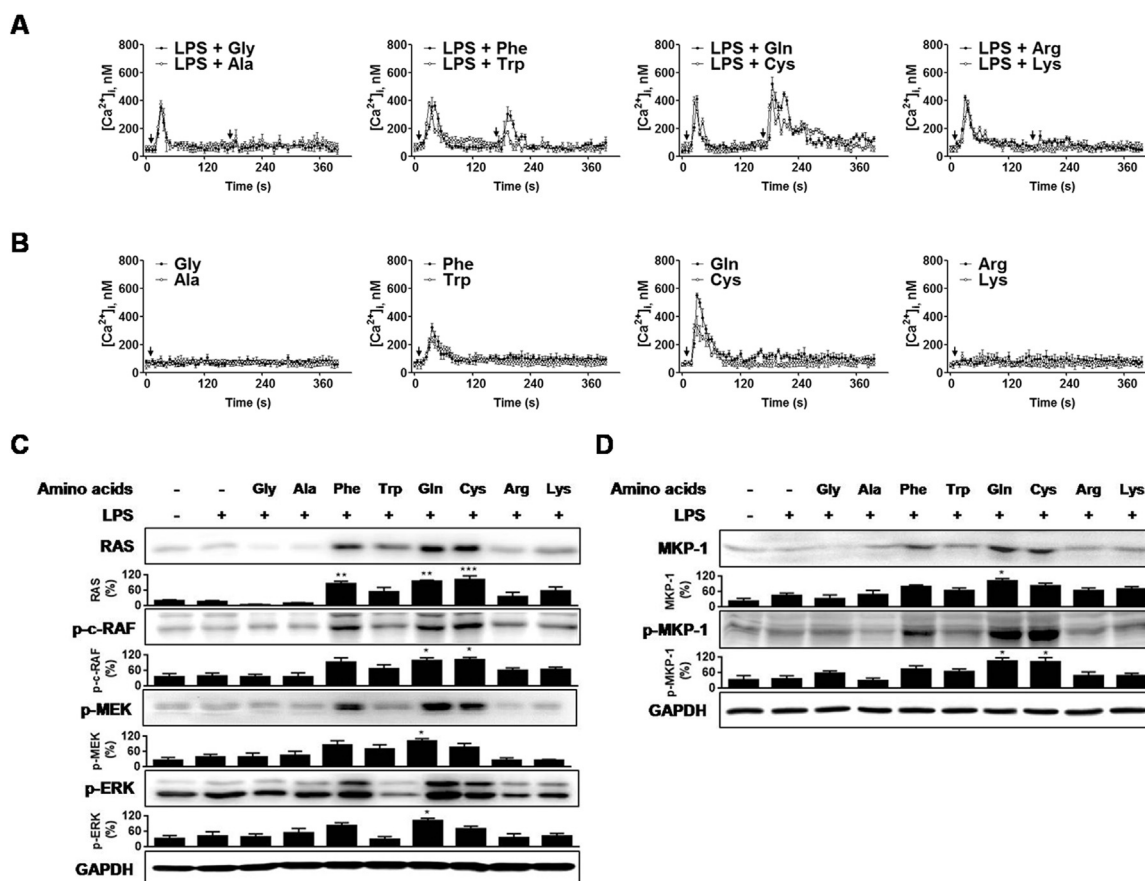
Fig. 4. Effects of L-amino acids on $[Ca^{2+}]_i$ and the Ras/ERK/MKP-1 pathway. (A) and (B) L-amino acids-induced $[Ca^{2+}]_i$ with or without LPS (1 ng/ml). MHS cells (2×10^6) were loaded with fluorescence probe Fluo 4/AM. (C) and (D) L-amino acids (40 mM) were added MHS cells (2×10^6) 5 min after LPS (1 ng/ml) stimulation. Cells were cultured for 15 min A representative of three independent experiments is shown. Each point represents mean $\pm$ S. E. of $[Ca^{2+}]_i$(A and B).

and Cys) was effective in inducing Ca^{2+} mobilization among the amino acids tested, and aromatic group (Phe and Trp) also induced Ca^{2+} response, but these responses were significantly smaller than that evoked by Gln.

We next compared the effects of amino acids on activation of ERK cascade and MKP-1 induction. Gln, Cys, and Phe, which showed Ca^{2+} provoking activities in the presence of LPS, activated the ERK cascade (Fig. 4(C)) and potentiated MKP-1 inductionand MKP-1 phosphorylation(Fig. 4(D)).

3.5. Comparison of other amino acids in their activities to suppress contact dermatitis (CD)

After 2, 4-dinitrofluorobenzene (DNFB) challenge, Gln and seven other amino acids (4% in saline) were topically applied on the ears of mice three times at 5, 10, and 15 min MKP-1 induction in the ears was assessed. Gln and Cys-induced MKP-1 induction, albeit to a lesser degree in the latter case. In contrast to *in vitro*, Trp failed to trigger MKP-1 induction (Fig. 5(A)). Application of DNFB to the ears increased ear swelling response (ESR) by approximately 120% compared with ears of mice that were treated with vehicle. Among the eight amino acids, only Gln and Cys significantly inhibited not only DNFB-induced ESR measured at 24 h (Fig. 5(B)), and marked spongiosis and extensive leukocyte infiltration in the swollen dermis (Fig. 5(C)), but also protein levels of interferon-gamma (IFN-γ), interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α) in the ears (Fig. 5(D)).

MKP-1 dependency of Gln and Cys suppression of CD was assessed. MKP-1 small interfering RNA (siRNA) significantly abrogated the Gln inhibition of ear inflammation (Fig. 6(A)-(B)) as well

as ear levels of cytokines (Fig. 6(C)). In contrast, MKP-1 siRNA did not significantly affect Cys-mediated inhibition of ear inflammation (Fig. 6(A)-(B)) and ear levels of cytokines (Fig. 6(C)). Given that one of mechanisms of Gln inhibition of inflammation is cPLA$_2$ inhibition [7,8,14], effects of Gln and Cys on cPLA$_2$ phosphorylation were examined. Gln or Cys application after (DNFB) challenge resulted in inhibition of cPLA$_2$ phosphorylation (Fig. 7(A)). However, whereas Gln-induced inhibition was reversed by MKP-1 siRNA, Cys-induced inhibition was not (Fig. 7(B)). Likewise, although Gln and Cys exerted similar inhibitory activities against the levels of a cPLA$_2$ metabolite, leukotriene B$_4$(LTB$_4$), in the ear tissues, MKP-1 siRNA abrogated the activity of Gln, but not that of Cys (Fig. 7(C)). Taken together, among the amino acids tested, only Gln suppressed CD in a MKP-1-dependent manner.

4. Discussion

Gln was previously found to show a strong anti-inflammatory activity via ERK-dependent MKP-1 induction. In this study, we found that this appears to be attributable to its ability to trigger sequential events – the steps that increase Ca^{2+} mobilization, followed by activation of Ras → ERK pathway.

Given that Gln-mediated MKP-1 induction is ERK-dependent [11,13], we investigated whether Gln can potentiate ERK signaling. Gln increased ERK phosphorylation and activity. MKP-1 phosphorylation and protein induction occurs as early as 5 min after Gln administration. Similarly, increases of ERK phosphorylation and activity by Gln were seen within 5 min, suggesting that increase of ERK activity resulted in MKP-1 induction.
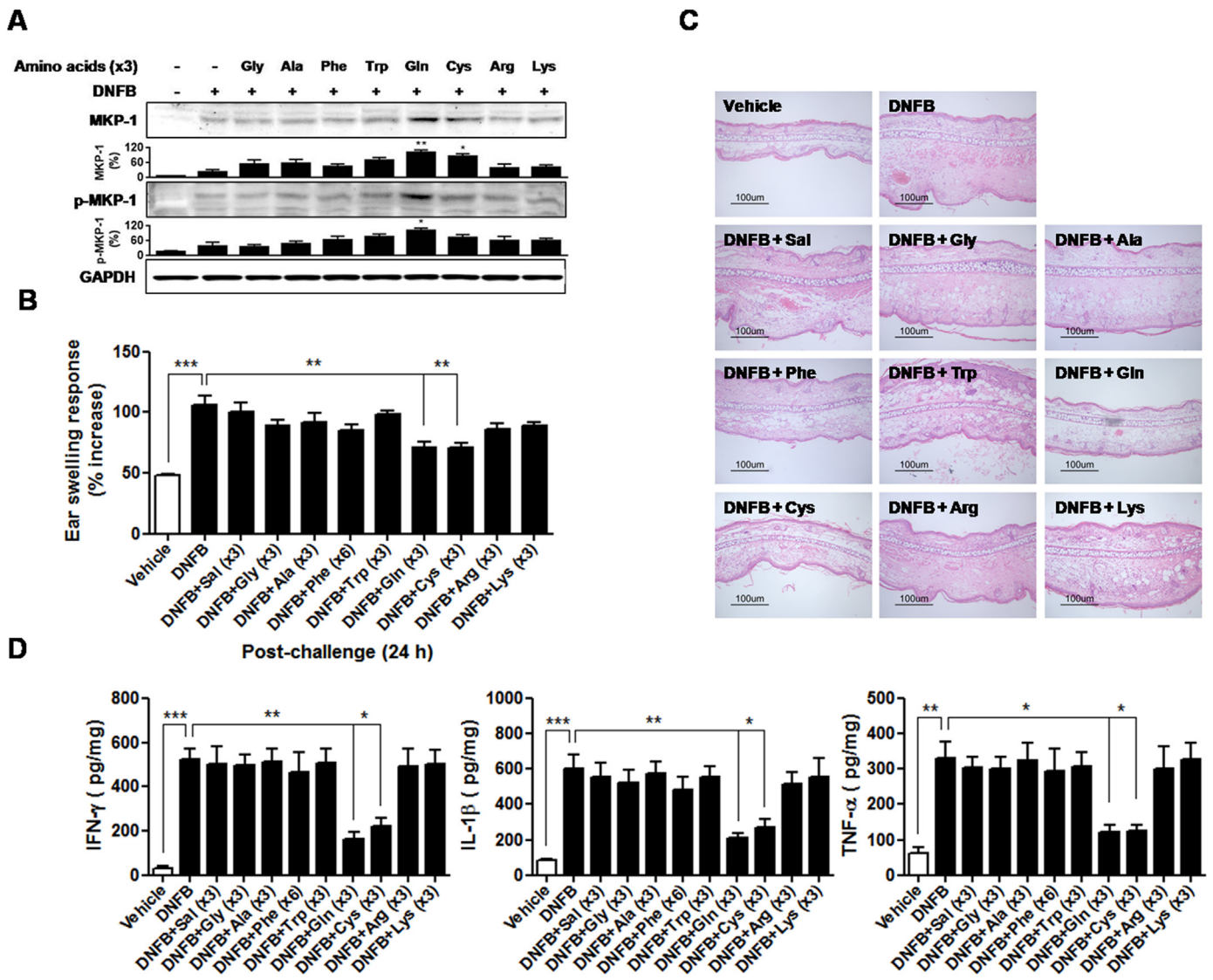
Fig. 5. Effects of amino acids with enhancing $[Ca^{2+}]_i$ on suppression of CD. L-amino acids (4% in saline) were topically applied onto the ears three times at 5, 10, and 15 min (A) Ears were removed at 30 min after DNFB challenge. A representative of five independent experiments with 3–5 mice/time point/experiment is shown. (B) ESRs were measured at 0 and 24 h after challenge. (C) H&E staining of the ear sections (scale bar =100 μm). (D) Cytokine levels in the ear homogenates. Data in (B) and (D) represent the mean ± standard deviation of three independent experiments with five mice per group. *$P < 0.05$;**$P < 0.01$;***$P < 0.001$.

How can Gln exert such effects? Extracellular stimuli such as growth factors, serum, hormones, and cytokines lead to activation of ERK MAPK through a signaling cascade [25,36–41]. The signaling via this cascade is usually initiated by the activation of cell surface small G proteins, Ras [42], which is localized to the inner leaflet of the plasma membrane and transmit the signal by recruiting Raf kinases (A-Raf, B-Raf, and C-Raf) to the plasma membrane, where they can be activated. Activated Raf, in particular C-Raf (formally Raf-1) binds to and phosphorylates the down-stream dual specificity kinases MEK1 and MEK2, which, in turn, phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr motif in their activation loop (known as the ERK cascade)[25,26,39,43,44]. Therefore, we explored whether Gln can activate the upstream kinases of ERK. Gln increased the levels of Ras protein and phosphorylation of, c-Raf, and MEK, indicating that Gln activates Ras-ERK pathway. In this study, we employed anti-phospho-c-Raf (Ser[338]) to examine Raf activation. However, given the complexity of Raf regulation by homo-and hetero-dimerisation as well as phosphorylation [45], we can not completely rule out the role of other Raf kinase.

What is the possible mechanism by which Gln activates Ras? In addition to a variety of extracellular stimuli leading to activation of

the ERK cascade described above, a linkage between Ras activation and Ca^{2+} has been described reported in neurons [32–35,46]. One of mechanisms is that these Ca^{2+} signals are mediated by Ca^{2+}/calmodulin (CaM) protein kinase II (CaMKII), a ubiquitous serine/threonine protein kinase that is activated by Ca^{2+} and CaM to phosphorylate diverse substrates involved in metabolism, neurotransmitter release and cell cycle control. For example, the activation of the Ras/Raf/MEK/ERK signal pathway by calcium/calmodulin is well established [47]. These observations led us to explore possible cross-talk between these two signal pathways. Consistent with these findings, we demonstrated that Gln evoked Ca^{2+} mobilization regardless of the presence of LPS in MHS macrophages. Gln also stimulated significant Ca^{2+} response in the absence of extracellular Ca^{2+}, suggesting that it mainly triggers the release of Ca^{2+} from an intracellular store. Ionomycin was comparable to Gln in terms of activation of Ras → ERK and MKP-1 induction *in vitro*. However, both Gln and ionomycin failed to activate the reactions in the absence of LPS, indicating that just Ca^{2+} increases are not sufficient to trigger such reactions. Ca^{2+} blocker abrogated not only Gln-mediated Ca^{2+} mobilization as well as the ERK cascade activation, but also Gln-mediated MKP-1
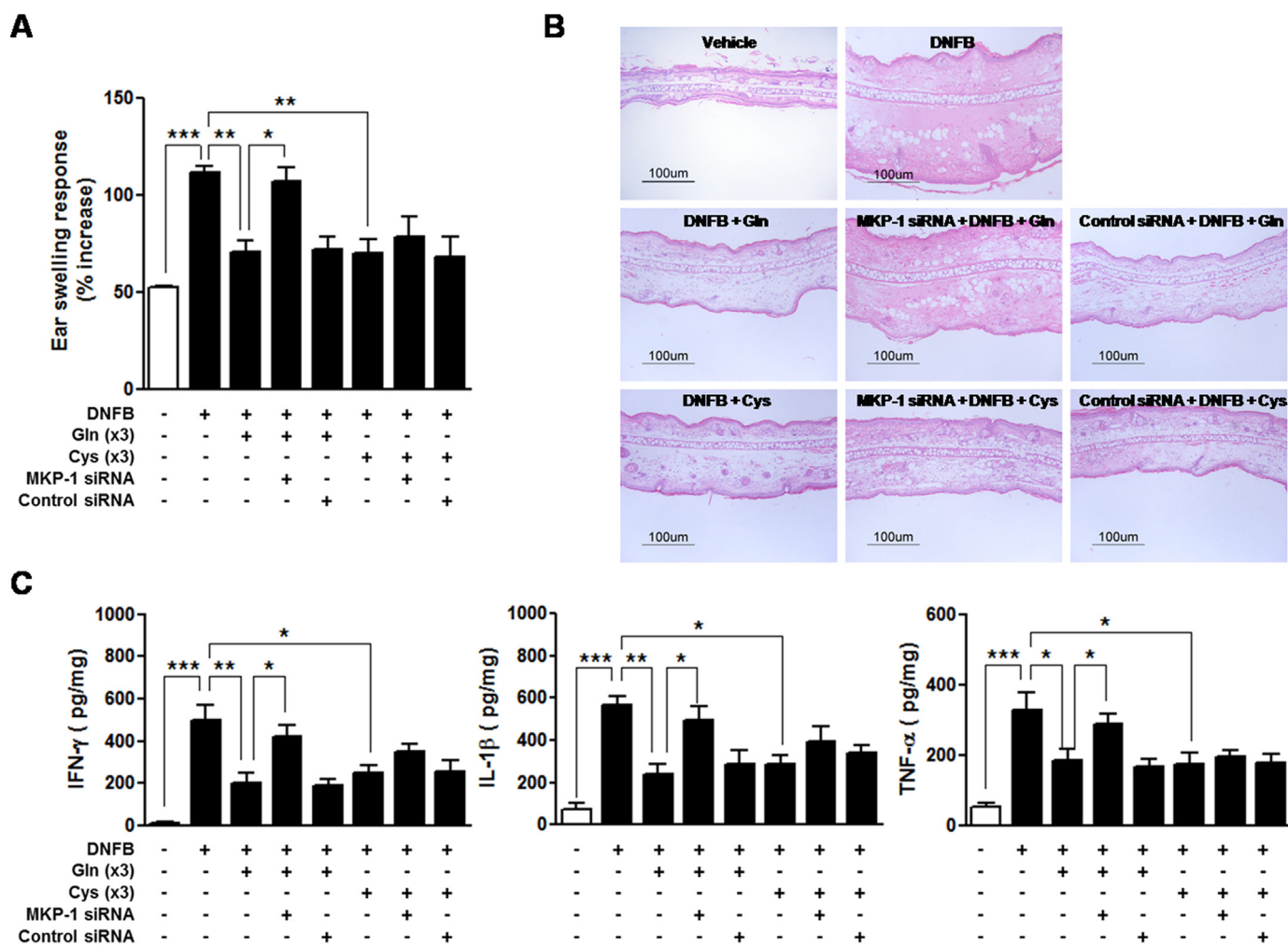
Fig. 6. MKP-1 siRNA abrogates suppressive activity of Gln against DNFB-induced CD. Mice were i.v. injected with 200 μl of the PEI mixture containing 0.4 nmol siRNA 24 h before challenge of DNFB. Gln or Cys was applied three times (×3) in the ear skin after DNFB challenge. (A) ESRs were measured at 0 and 24 h after challenge. (B) After measurement of ESR at 24 h, the mice were sacrificed, and the ear sections were stained with H&E (scale bar = 100 μm). (C) Measurement of cytokine levels in the ear tissues were performed. Data in (A) and (C) represent the mean $\pm$ standard deviation of three independent experiments with five mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.

phosphorylation and protein induction. It has been reported that L-amino acids, particularly aromatic amino acids enhance the stereoselective sensitivity of the CaR to its agonists [30]. However, Gln was the most effective in this study. This may be attributed to the differences in the concentrations of amino acids (2–3 mM vs. 20–40 mM in this study) or cell type (human embryonic kidney cell vs. mouse macrophage cell). Taken together, our data indicate that Gln raises $[Ca^{2+}]_i$ concentration and activates Ras → ERK pathway, leading to MKP-1 phosphorylation, which prevents the degradation of MKP-1 protein from ubiquitin/proteasome pathway, resulting in early and/or sustained induction of MKP-1.

Regarding the effects of other amino acids on triggering the Ca^{2+} → MKP-1 pathway, three amino acids – Gln, Cys (polar, uncharged) and Phe (aromatic) – were capable of mobilizing Ca^{2+} and activation of ERK cascade and MKP-1 induction in the presence of LPS in MHS cells, indicating that Ca^{2+} mobilizing activity is closely associated with its activity to trigger Ras-ERK-MKP-1 pathway. Interestingly, only the amino acids with Ca^{2+} mobilizing activity (Gln, Cys, and Phe) were able to activate Ras-ERK-MKP-1 pathway, indicating that, besides Gln, other amino acids including Cys and Phe were capable of MKP-1 induction via Ca-dependent activation of ERK cascade in vitro.

In contrast to the findings in vitro, Gln was the only amino acid to trigger MKP-1 induction significantly in a murine model of CD. Furthermore, only Gln and Cys significantly suppressed CD to a

similar extent in terms of ear inflammation and ear levels of cytokines. Interestingly, Gln and Cys also similarly inhibited DNFB challenge-induced $cPLA_2$ phosphorylation and the levels of a $cPLA_2$ metabolite, LTB_4, in the ear. When we assessed MKP-1 dependency of their suppressions using MKP-1 siRNA, MKP-1 siRNA reversed all the Gln's suppressive activities, confirming our previous findings [11–13], whereas Cys's suppressive activities were not significantly affected by MKP-1 siRNA. Therefore, our data indicate that Cys operates other mechanism rather than a mechanism mediated by MKP-1 in suppression of CD.

Ca^{2+}-Ras pathway is activated by a great variety of extracellular stimuli, including growth factors, hormones, and cell? extracellular matrix contacts [48]. Guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs) form one of the largest protein families found in nature, thus, representing the largest family of cell-surface receptors [49,50]. Many GPCRs possess distinct allosteric sites that can be targeted by exogenous substances to modulate the receptors' functions. These allosteric modulators include a variety of amino acids, ions, lipids, peptides, and accessory proteins [51]. Therefore, it is possible that, besides CaR [30,31], Gln binds to allosteric sites on GPCR, resulting in increasing intracellular Ca^{2+} via activation of the known phospholipase C/ inositol 1,4,5-trisphosphate pathway. The identification of the Gln-binding GPCR requires further study.
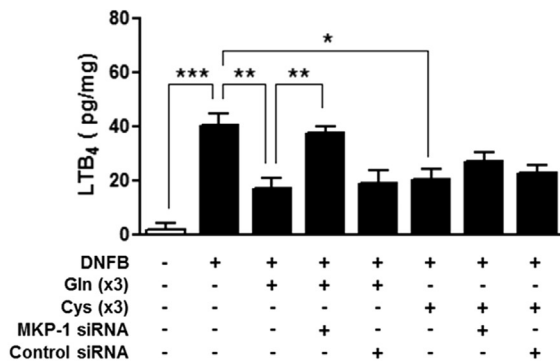
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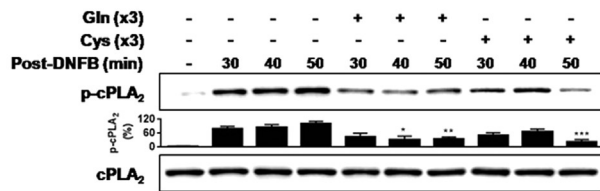
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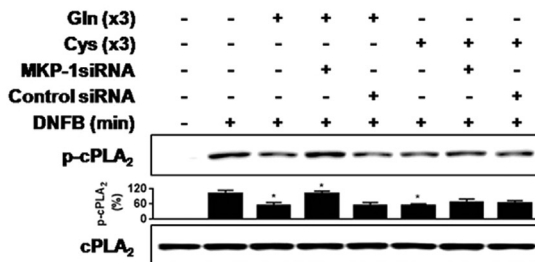
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Fig. 7. Gln and Cys inhibit cPLA$_2$ phosphorylation, but MKP-1 siRNA abrogates only Gln-induced inhibition.(A) The ears were removed at the indicated time points after DNFB challenge. Gln or Cys was applied three times ($\times$ 3) in the ear skin after DNFB challenge. A representative of three independent experiments with 3–5 mice/time point/experiment is shown. (B) The ears were removed at 30 min post challenge. (C) LTB$_4$ level in the ear samples at 24 h after DNFB challenge were calculated as pg of protein/mg of homogenized tissue solution. Data in (C) represent the mean $\pm$ standard deviation of three independent experiments with five mice per group. *P < 0.05;**P < 0.01;***P < 0.001.

5. Conclusions

We have previously demonstrated that Gln deactivates the two important enzymes involved in inflammatory reactions, p38 and cPLA$_2$, by a rapid induction of MKP-1 protein in an ERK-dependent way. In this study, we demonstrate that 1) Gln induction of MKP-1 is attributed to its ability to enhance Ca^{2+} and subsequently activate the Ras/c-Raf/MEK/ERK pathway, and 2) Gln is the only amino acid to suppress CD in an exclusively MKP-1-dependent manner.

This study provides important information to improve our understanding of the mechanisms underlying Gln induction of MKP-1 and to establish an easier approach to therapeutic strategies for a variety of human inflammatory diseases as steroid-replacing and/or steroid-sparing agent.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.05.011.

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