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Glutamine Suppresses Airway Neutrophilia by Blocking Cytosolic Phospholipase A2 via an Induction of MAPK Phosphatase-1

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Neutrophils are inflammatory cells that may contribute in a crucial way to the pathophysiology of steroid-resistant severe asthma. We previously reported that the nonessential amino acid L-glutamine (Gln) suppressed the recruitment of neutrophils into the airway in a murine model of asthma. In this study, we investigated the mechanisms by which Gln exerts beneficial effects in airway neutrophilia. We used the model we previously developed, which is suitable for examining sequential early asthmatic events, including neutrophil infiltration. Gln suppressed airway neutrophilia in a CXC chemokine-independent way. Airway neutrophilia was associated with cytosolic phospholipase A2 (cPLA2) and 5-lipoxygenase (5-LO) activities. p38 MAPK, the upstream pathway of cPLA2 and 5-LO, played a key role in inducing airway neutrophilia. Gln inhibited not only the phosphorylation of cPLA2 and p38 MAPK but also leukotriene B4 levels in the airways. Gln induced the early induction of MAPK phosphatase-1 (MKP-1) protein, a negative regulator of p38. MKP-1 small interfering RNA abrogated all the effects of Gln. Our results suggest that pathways involving p38/cPLA2/5-LO have a major role in airway neutrophilia. Gln suppresses airway neutrophilia via inhibiting p38 MAPK and its downstream pathways in an MKP-1-dependent way, which may provide a novel therapeutic strategy for pulmonary neutrophilic inflammatory diseases. The Journal of Immunology, 2012, 189: 5139–5146.
by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School, Chonbuk, Republic of Korea.

Reagents
L-Gln (Biotechnology performance certified, G-8540) and alanine (Ala) were purchased from Sigma-Aldrich (St. Louis, MO). Gln and Ala were dissolved in sterilized distilled water to reach 4%, the saturated concentration at room temperature, and 0.5 ml (∼1000 mg/kg) was administered i.p. (21). The cPLA2 inhibitor arachidonoyl trifluoromethyl ketone (AACOCF3; Merck KGaA, Darmstadt, Germany) was administered i.p. (20 mg/kg) 30 min before the second airway challenge (21). The 5-LO inhibitors AA-861 and MK-886 were purchased from Sigma-Aldrich and Cayman Chemical Company (Ann Arbor, MI), respectively. AA-861 and MK-886 were administered 1 h before the secondary airway challenge s.c. and i.p., respectively. We determined the optimum injection time in preliminary experiments. Leukotriene B4 (LTB4) was purchased from Cayman Chemical Company. The p38 MAPK inhibitor SB202190 and ERK inhibitor PD98059 from Calbiochem (San Diego, CA) were dissolved with DMSO. SB202190 (5 mg/kg) and PD98059 (12.5 mg/kg) were injected i.p. twice (48 h and 24 h before) and 30 min before the secondary airway challenge, respectively. Abs against cPLA2, phospho-cPLA2, MAPKs, phospho-MAPKs, and MAPK phosphatase-1 (MKP-1) were purchased from Cell Signaling Technology (Danvers, MA).

Immunization and challenge
Mice were immunized and challenged as described previously (23), with some modification. In brief, mice were immunized i.p. with 20 µg chicken egg OVA (OVA, grade V; Sigma-Aldrich) plus 1.0 mg aluminum hydroxide adjuvant (Inject Alum; Pierce, Rockford, IL) on day 0, and with OVA alone on day 14. The immunized mice were exposed to 1% aerosolized OVA for 20 min on days 28 and 35. Control animals received the same immunization and the first OVA airway challenge but were exposed to aerosolized saline instead of OVA for the second airway challenge.

Bronchoalveolar lavage
Bronchoalveolar lavage (BAL) was performed at the time indicated after the second airway challenge, as described previously (21, 23).

Immunoblotting analysis
Mice were sacrificed by cervical dislocation, and the lungs were collected immediately thereafter, briefly washed with cold PBS, and dried with blotting paper. The isolated lung tissues were frozen in liquid nitrogen and stored in −70°C until analysis. Small lung specimens were homogenized in the PhosphoSafe Extraction Reagent (Novagen, Madison, WI) and subjected to immunoblotting analysis, as described previously (20).

Cytokine assay
The levels of MIP-2, keratinocyte-derived chemokine (KC), and CXCL5 were quantified in BAL fluid using an ELISA performed according to the protocol of the manufacturer. The lower limits of detection for the cytokines were as follows: MIP-2 assay (≥1.5 pg/ml; R&D Systems, Minneapolis, MN), KC assay (≥2.0 pg/ml; R&D Systems), and CXCL5 (≥31.2 pg/ml; Abcam, Cambridge, U.K.).

Measurement of leukotriene B4
The level of LTB4 in BAL fluid was quantified using competitive ELISA performed according to the protocol of the manufacturer (Cayman Chemical Company). Sensitivity was 13 pg/ml.

Small interfering RNA interference
Small interfering RNA (siRNA) strands for cPLA2 and controls were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). In vivo delivery of siRNA was performed using in vivo-jet polyethylenimine (PEI; Polyplus-transfection, Illkirch, France), according to the instructions of the manufacturer. In brief, cPLA2 siRNA and PEI dissolved in 5% glucose were mixed in a volume of 200 µl for i.v. injection at room temperature for 20 min, and the mixture was administered 24 h before the secondary airway challenge. The mixture containing control siRNA and PEI dissolved in 5% glucose without siRNA were used as controls. To confirm that the cPLA2 siRNA used really blocked the synthesis of its target, an immunoblotting analysis was performed.

Statistical analysis
Data are presented as mean ± SEM. Student unpaired t test was applied to determine significant differences between corresponding treated groups and control groups. A p value < 0.05 was considered statistically significant.

Results
Gln inhibits airway neutrophilia in a CXC chemokine–independent manner
Secondary airway challenge with OVA resulted in increases in numbers of neutrophils in the airway, peaking at 8–12 h and declining thereafter. Gln was i.p. administered 20 min before or 20 min after the beginning of the secondary challenge. Gln significantly suppressed airway neutrophilia when it was administered after, not before, airway challenge (Fig. 1A). In addition, Gln suppressed neutrophil recruitment in a dose-dependent way (Fig. 1B). The suppression was not evident in the Ala control mice. On the basis of results, we used 4% of Gln and administered it at 20 min in subsequent experiments.

The next experiment examined the effects of Gln on BAL levels of the CXC chemokines MIP-2, KC, and CXCL5, which are principally chemotactic for neutrophils (24–26). After the secondary airway challenge, BAL levels of the three chemokines peaked at 2–4 h (Fig. 2A), but Gln was incapable of reducing the levels of the three chemokines (Fig. 2B). These results indicate that Gln did not affect the production of the important CXC chemokines in asthmatic lungs. Therefore, the subsequent experiment examined the possibility that Gln might affect the MIP-2– and KC-mediated chemotactic activity for neutrophils. Intratracheal (i.t.) instillation of MIP-2 and KC resulted in the accumulation of neutrophils in the airway in a dose-dependent manner, but the chemotactic activities of MIP-2 and KC were not inhibited by Gln at all (Fig. 2C, 2D). These data suggest that Gln uses a mechanism...
differing from that involving MIP-2 and KC in suppression of airway neutrophilia.

Involvement of cPLA2/5-LO/LTB4 in Gln suppression of airway neutrophilia

Given that Gln inactivates cPLA2 (19), we examined whether cPLA2 was associated with the Gln-mediated suppression of airway neutrophilia. We first examined the role of cPLA2 in airway neutrophil infiltration, using the cPLA2 inhibitor AACOCF3 and cPLA2 siRNA. AACOCF3 suppressed airway neutrophilia in a dose-dependent manner (Fig. 3A). Mice were i.v. administered with 0.4 nmol of cPLA2 siRNA or control siRNA 24 h before the secondary airway challenge. cPLA2 siRNA, but not control siRNA, significantly abrogated cPLA2 protein as well as the airway challenge–mediated phosphorylation of cPLA2 (Fig. 3B). cPLA2 siRNA, but not control siRNA, suppressed airway neutrophilia (Fig. 3C).

Activated cPLA2 is required for generation of various eicosanoids, such as LTs, PGs, and platelet-activating factor (27, 28). Among these, LTB4 is generated by 5-LO and acts as a potent neutrophil chemoattractant (29). Therefore, it is possible that Gln-mediated inhibition of cPLA2 activity leads to downregulation of such 5-LO metabolite levels, resulting in a decrease in neutrophil recruitment. To examine the involvement of 5-LO metabolites, we first looked at the effects of 5-LO inhibitors on neutrophil recruitment. The 5-LO inhibitors AA-861 and MK-886 significantly reduced neutrophil recruitment in a dose-dependent manner (Fig. 4A). BAL fluid levels of LTB4 were increased upon the secondary airway challenge, and reached a peak at 1 h (Fig. 4B), which was inhibited by Gln and cPLA2 siRNA (Fig. 4C). I.t. administration of LTB4 after the secondary airway challenge further increased the numbers of neutrophils in BAL fluids in a dose-dependent manner (Fig. 4D). Furthermore, i.t. LTB4 normalized BAL neutrophils in Gln-treated OVA-exposed mice (Fig. 4E).
These data clearly indicate that cPLA2/5-LO/LTB4 pathways play a key role in airway neutrophilia in this murine model of asthma.

**Gln inhibits the phosphorylation of cPLA2 and MAPKs**

We next examined the effect of Gln on cPLA2 phosphorylation in asthmatic lungs. Secondary airway challenge resulted in increases in cPLA2 phosphorylation, and administration of Gln after, not before, airway challenge resulted in an inhibition of cPLA2 phosphorylation (Fig. 5A). Ala did not show such inhibition. As the molecular mechanism of Gln inhibition of cPLA2 phosphorylation, we assessed the effect of Gln on phosphorylation of members of the MAPK family, including p38, JNK, and extracellular-signal-regulated kinase (ERK)1/2 (p42/p44), because activation of MAPK is a major upstream pathway for cPLA2 phosphorylation (30). Secondary airway challenge resulted in phosphorylation of all three MAPKs, and Gln inhibited the phosphorylation of p38 and JNK (Fig. 5B). Pretreatment of the mice with p38 inhibitor, SB202190, inhibited not only cPLA2 phosphorylation (Fig. 5C), but also airway neutrophilia (Fig. 5D). Additionally, SB202190 failed to reduce BAL fluid levels of CXC chemokines, but significantly reduced BAL LTB4 (Fig. 5E). These results suggest that Gln indirectly inhibits cPLA2 through deactivation of p38 MAPK.

**Gln-induced inhibition of p38 is associated with the early induction of MKP-1**

We have previously shown that Gln rapidly deactivates p38 by dephosphorylating them by rapid (~5 min) phosphorylation and protein induction of MKP-1 (20). To gain insight into the molecular mechanism of Gln inhibition of p38 phosphorylation, we examined the effect of Gln on MKP-1 phosphorylation and protein induction. Western blot analysis revealed that MKP-1 protein increased from 60 min and continued to rise over 120 min (Fig. 6A). The appearance of MKP-1 protein also correlated with the dephosphorylation of p38 seen in Fig. 5B. Gln administration resulted in an early induction of MKP-1. Administration of Gln immediately after cessation of the secondary airway challenge resulted in the appearance of MKP-1 as early as 10 min after Gln injection (Fig. 6A). Ala did not exert such an effect. This Gln-induced induction of MKP-1 also coincided with a Gln-induced dephosphorylation of p38, seen in Fig. 5B. To elucidate the role of MKP-1 in Gln-induced inhibition of p38 phosphorylation, we tried to block MKP-1 induction using MKP-1 siRNA. Mice were i.v. administered 0.2 or 0.4 nmol of MKP-1 siRNA or 0.4 nmol of control siRNA 24 h before the secondary airway challenge. MKP-1 siRNA, but not control siRNA, significantly abrogated the airway challenge–mediated induction of MKP-1 (Fig. 6B). In mice pretreated with 0.4 nmol of MKP-1 siRNA, Gln did not induce MKP-1 and did not inhibit p38 phosphorylation (Fig. 6C). These data clearly indicate that Gln inhibits phosphorylation of p38 through the early induction of MKP-1.

**MKP-1 siRNA attenuates Gln suppression of airway neutrophilia**

Using MKP-1 siRNA, we examined whether the ability of Gln to induce MKP-1 protein was associated with the beneficial effect of Gln on airway neutrophilia. Pretreatment of MKP-1 siRNA, but not control siRNA, abrogated the Gln-mediated suppression of airway neutrophilia (Fig. 7A) as well as BAL fluid level of LTB4 (Fig. 7B).

**ERK inhibitor rescues Gln-mediated induction of MKP-1 and suppression of neutrophil infiltration**

We finally examined the role of ERK in the actions of Gln, using the ERK inhibitor PD98059, as the involvement of ERK in this context has been mentioned previously (20). PD98059 nearly completely abrogated not only Gln induction of MKP-1 (Fig. 8A) but also Gln suppression of airway neutrophilia (Fig. 8B). These data indicate...
that Gln exerts MKP-1 induction and suppression of airway neutrophil infiltration in an ERK-dependent way.

**Discussion**

The study investigated the mechanism of actions of Gln in the suppressed recruitment of neutrophils into the airway. In this study, we used the previously developed protocol in which mice had twice received i.p. immunization, followed by two broncho-provocations with aerosolized OVA with a 1-wk interval (23). This model seems to be suitable for identifying the cascade events involved in an asthmatic reaction, including airway neutrophilia. As a possible mechanism, we first examined the ability of Gln to inhibit the production of the CXC chemokines. However, Gln did not display such activity. Furthermore, Gln failed to inhibit the abilities of KC and MIP-2 to recruit neutrophils into the airway. This finding suggests that another neutrophilic chemokine or signaling pathway that is blocked by Gln, rather than KC and MIP-2, is implicated in airway neutrophilia.

Importantly, we found that neutrophil recruitment was inhibited by cPLA2 inhibitors, such as AACOCF3 and cPLA2 siRNA, indicating that cPLA2 plays a key role in airway neutrophil sequestration in this murine model of asthma. Presently, Gln was able to inhibit cPLA2 phosphorylation in asthmatic lungs when it was administered after, but not before, the secondary airway challenge. This might explain our observations of the inhibitory effect of Gln against airway neutrophilia when it was administered

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**FIGURE 5.** Gln inhibits the phosphorylation of cPLA2 and p38, and p38 inhibitor inhibits cPLA2 phosphorylation and airway neutrophilia. (A and B) Gln and Ala were administered 20 min post challenge. SB202190 (5 mg/kg) was injected i.p., and (C) the levels of cPLA2 phosphorylation and (D) neutrophil numbers in BAL fluid were measured 30 min and 8 h after the secondary airway challenge, respectively. (E) SB202190 was injected i.p. and BAL levels of MIP-2, KC, CXCL5, and LTB4 were measured at 2 h. (A–C) A representative of three independent experiments with three to five mice per time point per experiment is shown. (D and E) Data represent the mean ± SD of two separate experiments (n = 5 per group). *p < 0.05 versus OVA group.

**FIGURE 6.** Gln-induced inhibition of p38 is associated with the early induction of MKP-1. (A) Gln and Ala were administered 20 min post challenge. (B) MKP-1 siRNA and control siRNA were administered i.v. 24 h before the secondary airway challenge. MKP-1 was measured at 90 min. (C) Then 0.4 nmol of siRNAs was given, and phospho-p38, phospho-cPLA2, and MKP-1 induction were measured at 30 min. A representative of two or three independent experiments with three to five mice per time point per experiment is shown. **p < 0.05 versus OVA group.
FIGURE 7. MKP-1 siRNA attenuates Gln suppression of airway neutrophilia and LTB4 levels. Effects of Gln and MKP-1 siRNA on the BAL fluid levels of (A) neutrophils and (B) LTB4. Neutrophils and LTB4 were measured at 8 h and 1 h, respectively. Data represent the mean ± SD of two separate experiments (n = 5 per group).

after, but not before, the secondary airway challenge. Although cPLA2 activity has been shown to be involved in neutrophilic sequestration in lung inflammation (31) as well as in neutrophil integrin expression for trans-endothelial migration (32), the role of cPLA2 in neutrophilic inflammation in asthma has not been well documented. In this regard, we presented evidence that cPLA2 is a key enzyme involved in neutrophilic inflammation in asthma.

Activation of the function of cPLA2 is mediated by Ca2+ and MAPK (33). In response to Ag stimulation, the intracellular Ca2+ level increases so Ca2+ can bind to the C2 domain of cPLA2. As a result of Ca2+ binding to the C2 domain, cPLA2 can be translocated from the cytosol to the cell membranes, including endoplasmic reticulum, Golgi apparatus, and perinuclear membranes (34). Activation of the hydrolizing function of cPLA2 requires inhibition of cPLA2 phosphorylation (19, 21) is due to its capability to inhibit the phosphorylation of MAPKs, which are the major upstream pathway for cPLA2 phosphorylation (35). We previously reported that Gln deactivates p38 and JNK MAPKs in LPS-injected mice (20). In this study, we also demonstrated that Gln inhibited the phosphorylation of p38 and JNK. Furthermore, pretreatment of mice with the p38 inhibitor SB202190 inhibited cPLA2 phosphorylation but also airway neutrophilia. These results further strengthen the idea that cPLA2 is critically involved in airway neutrophilia, and suggest that Gln indirectly inhibits cPLA2 phosphorylation through deactivation of, at least in part, p38 MAPK.

MKP-1 was originally identified as an ERK-specific phosphatase (36, 37), but has since been also recognized as being able to dephosphorylate and inactivate both p38 and JNK MAPKs, its substrate specificity being dependent on cell type and context (38, 39). As a result of its function in deactivating p38 MAPK, MKP-1 serves as an important negative regulator of inflammatory responses. MKP-1 is also induced by multiple immunosuppressive agents, including glucocorticoids and anti-inflammatory cytokines, and this induction partially mediates the inhibitory effects of these agents on MAPK activation (40). Induction of MKP-1 by TGF-β and IL-10 also contributes to the suppressive effects of these potent anti-inflammatory cytokines on the expression of genes encoding proinflammatory mediators (41, 42). Therefore, it appears that the induction of MKP-1 represents a common mechanism of immunomodulatory agents.

Gln-induced inhibition of p38 was attributed to the early induction of the dual phosphatase MKP-1 protein. This conclusion arose from two observations. First, Gln administration resulted in a rapid induction of MKP-1, which coincided with Gln-induced inhibition of p38. Second, MKP-1 siRNA abrogated the ability of Gln not only to induce MKP-1 induction and inhibition of p38 but also to suppress airway levels of neutrophils and LTB4. These findings might explain our previous observation that Gln-mediated inhibition of cPLA2 phosphorylation (19, 21) is due to its capability to inhibit the phosphorylation of MAPKs, which are the major upstream pathway for cPLA2 phosphorylation (30). In fact, we have previously shown that Gln rapidly deactivates p38 by dephosphorylating them only when these MAPKs were phosphorylated in response to LPS, and this effect of Gln was mediated by rapid (~5 min) phosphorylation and protein induction of MKP-1 (21). Therefore, a rapid and early induction of MKP-1, resulting in deactivation of p38 as well as cPLA2, seems to be a common important mechanism of the anti-inflammatory activity of Gln.

Activated cPLA2 catalyzes the production of arachidonic acid, which is converted by 5-LO into LTA4 and subsequently into LTB4, which acts as the most potent bioactive lipid chemoattractants and activators of neutrophils via the receptor for LTB4 (BLT1) expressed on neutrophils (29, 43). We found that the 5-LO inhibitors AA-861 and MK-886 significantly reduced neutrophil recruitment. Our kinetic study showed increases in LTB4 levels in the airways after second airway challenge. Gln significantly decreased the BAL levels of LTB4. Finally, i.t. instillation of LTB4

FIGURE 8. An ERK inhibitor abrogates (A) Gln induction of MKP-1 and (B) Gln suppression of airway neutrophilia. The ERK inhibitor PD98059 and Gln were administered 30 min before and 20 min after the secondary challenge, respectively. The number of neutrophils was determined at 8 h. Data represent the mean ± SD of two separate experiments (n = 5 per group).
cPLA2 has a key function in the induction of airway neutrophilia. Collectively, our data indicate that the cPLA2/5-LO pathway is important for neutrophil recruitment in asthmatic lungs. Therefore, Gln suppression of airway neutrophil recruitment seems to be attributed to its ability to inhibit the cPLA2/5-LO pathway. However, the ~70–80% inhibition of airway neutrophilia by Gln or inhibitors of cPLA2 and 5-LO suggests that other pathways independent of cPLA2 likely participated in airway neutrophil sequestration in this murine model of asthma.

It has been shown that IL-17 plays an important role in allergen-induced accumulation of neutrophils in the airway during the pathophysiological process of allergic asthma (44, 45). We examined the link between airway neutrophilia and IL-17. IL-17 peaked at around 18 h post challenge, and Gln reduced BAL fluid IL-17 (data not shown). However, as the number of airway neutrophils peaked at 8–12 h (Fig. 1A) and IL-17 was rarely detected until 8 h, IL-17 does not seem to play an important role in neutrophil sequestration in this murine model we used.

We demonstrated that the ERK inhibitor PD98059 blocked not only Gln-induced MKP-1 protein induction but also the suppressive activity of Gln against allergen-induced airway neutrophilia, indicating that the Gln action mechanism is an ERK-dependent process. ERK phosphorylates MKP-1 on two C-terminal serine residues—Ser359 and Ser364—which leads to stabilization of the protein via the prevention of its proteosomal degradation (46). Therefore, our findings, together with our previous report showing similar results in the LPS-induced septic shock model (20), suggest that Gln induces the early induction of MKP-1 protein by preventing its degradation through inducing MKP-1 phosphorylation in an ERK-dependent manner. Recently, we found that Gln increases ERK phosphorylation within 5 min, which is critical for early MKP-1 induction. We are currently investigating how Gln increases ERK phosphorylation.

In summary, we consistently found that the pathway involving cPLA2 has a key function in the induction of airway neutrophilia. Gln, which inhibited cPLA2 through MAPK inhibition, suppressed airway neutrophilia. Given that even the parenteral administration of Gln as a component of supportive nutritional therapy is safe (47, 48), Gln may provide a therapeutic approach to pulmonary neutrophilic inflammatory diseases, such as steroid-resistant severe asthma and chronic obstructive pulmonary diseases, and many other inflammatory diseases in which cPLA2 plays an important role.

Disclosures
The authors have no financial conflicts of interest.

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