RESEARCH ARTICLE

Phenethyl isothiocyanate suppresses nitric oxide production *via* inhibition of phosphoinositide 3-kinase/Akt-induced IFN-γ secretion in LPS-activated peritoneal macrophages

Toru Okubo[1], *Kazuto Washida*[2] *and Akira Murakami*[1]

[1] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan
[2] Nara Prefectural Small and Medium-sized Enterprises Support Corporation, Nara Prefectural Agricultural Experiment Station, Shijo, Kashihara, Nara, Japan

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Phenethyl isothiocyanate (PEITC), a constituent of many cruciferous vegetables, is well known to have versatile physiological activities, including chemopreventive effects. On the other hand, its anti-inflammatory effects are poorly reported. Nitric oxide (NO) is associated with a wide variety of inflammatory diseases. In this study, we investigated the effects of PEITC on NO production in LPS-activated peritoneal macrophages from ICR mice. The signaling pathway of LPS-induced NO production was examined using neutralizing antibodies [anti-interferon (IFN)-γ and anti-interleukin (IL-12)] and specific protein kinase inhibitors, as well as others. The activity of PEITC toward NOx production was assessed in mice that received LPS *via* intraperitoneal administration. The neutralizing antibody of anti-IFN-γ, but not anti-IL-12, suppressed LPS-induced NO production by 90%. LY294002, a specific inhibitor of phosphoinositide-3-kinase, suppressed Akt and *IFN-γ* mRNA expression up-regulated by LPS, whereas PEITC exhibited a similar inhibition profile. Furthermore, oral administration of PEITC significantly suppressed the serum concentration of NOx in ICR mice. Our results suggest that PEITC suppresses LPS-induced NO production *via* inhibition of Akt activation and the resultant decrease in expression of IFN-γ. This is one of the first reports to demonstrate a marked anti-inflammatory effect of PEITC following its oral administration.

Keywords:
Inflammation / Interferon-γ / LPS / Nitric oxide / Phenethyl isothiocyanate

1 Introduction

Nitric oxide (NO) is a small free radical that has many physiological functions, such as vasodilatation, neurotransmission, immunoregulation, and inflammation [1]. It is synthesized from L-arginine by three NO synthase (NOS) isoforms, *i.e.* endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). eNOS and nNOS are constitutively

Correspondence: Dr. Akira Murakami, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
E-mail: cancer@kais.kyoto-u.ac.jp
Fax: +81-75-753-6284

Abbreviations: AP-1, activator protein-1; **BUGLS,** 3-butenyl glucosinolate; **BUITC,** 3-butenyl isothiocyanate; **CREB,** cAMP-responsive element binding protein; **eNOS,** endothelial NOS; **GSTP1,** glutathione-*S*-transferase P1; **HPRT,** hypoxanthine-guaninephosphoribosyltransferase; **IκB,** inhibitor of NF-κB; **iNOS,** inducible NOS; **JAK,** janus kinase; **JNK,** c-Jun *N*-terminal kinase; **Mϕ,** macrophages; **MAPK,** mitogen-activated protein kinase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB,** nuclear-factor κB; **nNOS,** neuronal NOS; **NO,** nitric oxide; **NOS,** NO synthase; **PECs,** peritoneal exudates cells; **PEGLS,** phenethyl glucosinolate; **PEITC,** phenethyl isothiocyanate; **PI3K,** phosphoinositide 3-kinase; **pMϕ,** peritoneal macrophages; **PTEN,** phosphatase and tensin homolog deleted from chromosome 10; **STAT,** signal transducers and activators of transcription; **TLR4,** toll-like receptor 4; **TNF-α,** tumor necrosis factor-α

expressed in endothelial cells and neuronal tissues, respectively, whereas iNOS is induced in macrophages (Mϕ) and lymphocytes by various stimuli, such as bacterial endotoxin LPS and pro-inflammatory cytokines. In addition, this inducible isoform is capable of generating a large quantity of NO in a micromolar range, while NO production from eNOS and nNOS is constant and within the nanomolar range. The overproduction of NO is associated with inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and Alzheimer's disease [2].

The promoter region of the mouse *iNOS* gene contains several binding sites for transcription factors such as nuclear factor-κB (NF-κB), as well as activator protein-1 (AP-1), CCAAT-enhancer box binding protein, cAMP-responsive element binding protein (CREB), and the signal transducers and activators of transcription (STAT)1 [3]. NF-κB, STAT1, and AP-1 are required for *iNOS* gene expression in RAW264.7 Mϕ induced by LPS and pro-inflammatory cytokines [4–6]. On the other hand, LPS-induced iNOS expression is mediated by various cytokines, including IFN-α/β [7], IFN-γ [8], IL-12 [9], and tumor necrosis factor-α (TNF-α) [10] in an autocrine fashion in Mϕ from different strains of mice.

Park *et al.* suggested that phosphoinositide 3-kinase (PI3K) participates in the signaling pathway of LPS plus IFN-γ-induced iNOS expression [11]. PI3K is activated through binding of myeloid differentiation factor 88, an adaptor protein of Toll-like receptor 4 (TLR4) [12]. Activated PI3K phosphorylates the three-position hydroxyl group of the inositol ring of phosphatidylinositol in the cell membrane and then brings Akt to the plasma membrane. The PI3K/Akt signaling pathway positively regulates LPS-induced NO production in Mϕ [13]. Furthermore, PI3K and mammalian target of rapamycin mediate LPS-induced NO production *via* IFN-β, an autocrine co-factor in RAW264.7 cells [14].

Phenethyl isothiocyanate (PEITC, Fig. 1), a prominent constituent of many edible cruciferous vegetables such as watercress [15] and horseradish [16], is generated from phenethyl glucosinolate (PEGLS) by myrosinase in myrosine cells. PEITC has been well documented to have many remarkable activities, including induction of phase II detoxifying enzymes [17] and induction of apoptosis of cancer cells [18], as well as inhibition of angiogenesis [19]. However, its anti-inflammatory activities remain to be fully demonstrated. Although PEITC was reported to suppress iNOS, cyclooxygenase-2 [20], and IL-1β [21] expression in RAW264.7 Mϕ, the molecular mechanisms underlying these activities are still unknown. In addition, there is no known report on the effects of oral administration of PEITC on NO production in a mice model.

In this study, we investigated the molecular mechanisms underlying the effects of PEITC on LPS-induced NO production in murine primary peritoneal Mϕ (pMϕ) and evaluated its effects on NOx (nitrite+nitrate) production in mice injected intraperitoneally with LPS.

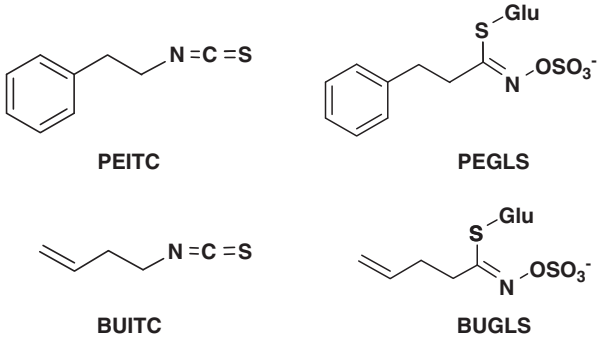

Figure 1. Chemical structures of PEITC, PEGLS, BUITC, and BUGLS.

2 Materials and methods

2.1 Mice

Four- and five-week-old ICR mice were purchased from Japan SLC (Shizuoka, Japan). All mice were quarantined for more than 1 week before starting the experiments, kept under specific pathogen-free conditions, and handled according to the Guidelines of the Regulation of Animals, as provided by the Experimentation Committee of Kyoto University, which approved the present animal experiments (♯19–38).

2.2 Chemicals

PEITC and 3-butenyl isothiocyanate (BUITC) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Both PEGLS and 3-butenyl glucosinolate (BUGLS) were purified using a method reported by Barillari *et al.* [22], with some modifications. *Barbarea verna* seeds were extracted with boiling water, then the extract was obtained by centrifugation, deproteinized by addition of 1 M Zn $(OAc)_2$, and subjected to additional centrifugation, after which the supernatant was subjected to chromatography with a DEAE-Sephadex A-25. The concentrated fraction was extracted with boiling methanol and the extract was centrifuged. The supernatant was added to chilled ethanol and PEGLS was obtained as a white powder after centrifugation. BUGLS was isolated from *Brassica rapa* L. (*Yamato-mana*) seeds in the same manner as described above. LPS (*Salmonella enterica* serotype *typhimurium*) was purchased from Sigma-Aldrich (St. Louis, MO). SP60012 was from Tocris Cookson (Bristol, UK), and AG490 and PD98059 were obtained from Calbiochem (LaJolla, CA). Dulbecco's modified eagle medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). Antibodies against the inhibitors of NF-κB

(IκB)α (sc-371) and phospho-janus kinase (JAK)2 (Tyr1007/Tyr1008, SC-16566-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-Akt (Ser473, ♯9271), Akt (♯9272), phospho-phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (Ser380, ♯9551), phospho-c-Jun *N*-terminal kinase (JNK) (Thr183/Tyr185, ♯9251), phospho-CREB (Ser133, ♯9191), JAK2 (♯3773), phospho-STAT1 (Tyr701, ♯9171), and STAT1 (♯9172) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against mouse IFN-γ were from Endogen (Wobum, MA), mouse IL-12 from eBioscience (San Diego, CA), and control rat IgG from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless specified otherwise.

2.3 Isolation and culture of murine primary pMϕ

To isolate pMϕ, ICR mice were killed by cervical dislocation and each peritoneal cavity was washed twice with 5 mL of Dulbecco's modified eagle medium medium supplemented with 10% fetal bovine serum and containing 1% heparin sodium. Peritoneal exudate cells were harvested and centrifuged at $3000 \times g$ for 5 min at 4°C, then seeded and incubated overnight. After washing the plates twice with PBS, adherent pMϕ were obtained and used in the experiments.

2.4 Measurements of nitrite and IFN-γ concentration

NO production was determined based on the amount of nitrite, a stable end-product of NO. Nitrite concentrations in media supernatants were determined by a Griess reaction. pMϕ (5×10^5 cells/200 μL) in 96-well plates were treated with or without PEITC, BUITC (2∼10 μM, each), PEGLS, or BUGLS (50 μM, each) for 30 min before exposure to LPS (500 ng/mL) for 48 h. Each supernatant (100 μL) was mixed with 100 μL of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamide dihydrocholide in distilled water]. Absorbance was measured at 540 nm. The concentration of IFN-γ was quantified using an ELISA kit (Endogen® Mouse IFN-γ ELISA Kit, Pierce Biotechnology) according to the manufacturer's instructions. The media were used without dilution.

2.5 Cell viability

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The value for cell viability of the positive control cells, which were treated with 0.5% DMSO and LPS, was standardized as 100%.

2.6 RT-PCR

pMϕ (5×10^6 cells/2 mL) were cultured with or without PEITC in 12-well plates for 6 (*IFN-γ*) or 18 h (*iNOS*). Total RNA was extracted from the cells using TRIzol® Reagent. *Hypoxanthine-guanine phosphoribosyl transferase* (*HPRT*) transcripts served as internal controls. cDNA was synthesized using an RNA PCR Kit (TaKaRa Bio, Shiga, Japan) with 1 μg of total RNA. PCR amplification was then performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA). The PCR conditions consisted of 30 cycles for 40 s at 90°C, 40 s at 62°C, and 60 s at 72°C for *IFN-γ*, with 29 cycles for 20 s at 95°C, 25 s at 55°C, and 45 s at 72°C for *iNOS*, or 31 cycles for 30 s at 95°C, 25 s at 55°C, and 45 s at 72°C for *HPRT*. The sequences of these primers are summarized in Table 1.

2.7 Western blot analysis

pMϕ (5×10^6 cells/2 mL) were cultured with or without PEITC (8 μM) or with neutralizing antibodies (10 μg/mL) in 12-well plates for 1 or 3 h, respectively, and then washed twice with PBS and lysed in lysis buffer [10 nM Tris, pH 7.4, 1% sodium dodecyl sulfate, 1 mM sodium metavanadate (V)] and centrifuged at $3200 \times g$ for 5 min. Denatured proteins (40 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h, the membrane was treated with the appropriate specific primary antibody (1:1000), followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:1000). The blots were developed using ECL Western blot detection reagents (GE Healthcare, Buckinghamshire, UK).

Table 1. Primers used for RT-PCR

Gene	Primer	Sequence (5′ to 3′)	Product size (bp)
IFN-γ	Forward	AACgCTACACACT gCATCTTgg	237
	Reverse	gACTTCAAAgAg TCTgAgg	
iNOS	Forward	CTgCAgCACTTggATC AggAACCTg	311
	Reverse	gggAgTAgCCTgTgTg CACCTggAA	
HPRT	Forward	gTAATgATCATTCAA CgggggAC	196
	Reverse	CCAgCAAgCTTgCAA CCTTAACCA	

2.8 Animal treatments

A total of 24 five-week-old male ICR mice were divided into one control and five experimental groups, with four mice in each group. Group 1 served as an untreated control and Group 2 mice were given a normal diet. Mice in Groups 3–6 were given PEITC, PEGLS, BUITC, or BUGLS (7.5 mmol/kg each), respectively, in the basal diet from the beginning of the experiment for 44 h. The mice in Groups 2–6 were injected intraperitoneally with LPS (1 mg/kg) at 24 h after starting the experiment and blood samples were collected 20 h after LPS injection, and then centrifuged at 3000 × *g* for 15 min at room temperature to prepare serum samples, which were stored at −80°C until analysis. This experiment was performed twice.

2.9 Measurement of NOx and IFN-γ in serum

The serum samples were mixed with methanol (1:2, v/v) to precipitate the proteins, and then centrifuged at 21 000 × *g* for 10 min at room temperature. An NOx analyzing system (ENO-20, EICOM, Kyoto, Japan) was used to analyze the concentration of NOx in the supernatant. IFN-γ in serum was quantified as described above.

2.10 Statistical analysis

Data are shown as the mean ± standard deviation (SD) from three independent experiments, unless specified otherwise, and statistical significance was evaluated by Student's *t*-test. Differences were considered significantly at $p < 0.05$.

3 Results

3.1 PEITC suppresses NO production and iNOS mRNA expression in pMϕ

To investigate the effects of PEITC on LPS-induced NO production, the concentrations of nitrite in the culture supernatants were measured using Griess assays. pMϕ were treated with the vehicle, PEITC, BUITC (2 or 10 μM each), PEGLS, or BUGLS (50 μM each) 30 min prior to LPS stimulation for 48 h. As shown in Fig. 2A, LPS notably increased the concentration of nitrite by 8-fold as compared with the vehicle control, and both PEITC and BUITC (10 μM) suppressed it (89 and 87% inhibition, respectively, $p < 0.05$) without noted cytotoxicity (Fig. 2B). In contrast, PEGLS and BUGLS (each 50 μM) showed no inhibition. To clarify how PEITC attenuates NO production, *iNOS* mRNA expression was examined by RT-PCR analysis. As shown in Fig. 2C, treatment with PEITC (10 μM), but not PEGLS (50 μM), for 8 h abolished LPS-induced *iNOS* mRNA expression.

3.2 IFN-γ a critical autocrine mediator for NO induction

LPS-induced iNOS expression and resultant NO production in Mϕ were reported to be mediated by several inflammatory cytokines, including IFN-γ [8] and IL-12 [9]. To examine which cytokines are involved in NO production in LPS-challenged pMϕ from ICR mice, the effects of IL-12 and IFN-γ neutralization antibodies on NO production were assessed (Fig. 3A). The anti-IFN-γ neutralization antibody, but not control IgG or the anti-IL-12 antibody, substantially suppressed LPS-induced NO production by 90% ($p < 0.05$). It is also important to note that recombinant IFN-γ is capable of producing NO, the potency of which is comparable to that of LPS.

3.3 JAK2, PI3K/Akt, and JNK1/2/3 signaling pathways associated with NO production

Okugawa *et al.* documented that various signal molecules, including JAK2, PI3 K, and JNK1/2/3, are involved in LPS-induced NO production in RAW264.7 Mϕ [23]. In this study, we examined the involvement of these molecules in NO production in pMϕ using specific protein kinase inhibitors. Their concentrations in cell culture media were determined to be the non-lethal maximum, as detected by an MTT assay (data not shown). AG490 (a specific inhibitor of JAK2), LY294002 (PI3 K), and SP600125 (JNK1/2/3) showed substantial suppression of LPS-induced NO production (Fig. 3B). In contrast, PD98059 (MEK1) and SB203580 [p38 mitogen-activated protein kinase (p38 MAPK) significantly augmented the production by 1.5- and 2.1-fold, respectively.

3.4 PEITC suppresses IFN-γ secretion and its mRNA expression

Next, we examined the effects of PEITC on LPS-induced IFN-γ secretion and *IFN-γ* mRNA expression. As shown in Fig. 4, LPS dramatically increased IFN-γ protein concentration by 6.0-fold, as detected by ELISA, whereas PEITC (8 μM) suppressed it by 88% ($p < 0.05$). In addition, both AG490 (20 μM) and LY294002 (4 μM), but not SP600125 (4 μM), showed significant suppression by 86 and 80% ($p < 0.05$), respectively, and reduced the level of *IFN-γ* mRNA expression in similar manners.

3.5 PEITC suppresses LPS-induced Akt activation

Since the PI3K/Akt signaling pathway was suggested to be associated with *IFN-γ* mRNA expression (Fig. 4), the effects of PEITC on LPS-induced Akt phosphorylation at Ser473 were investigated. PEITC (8 μM) inhibited Akt phosphorylation 1 h after LPS stimulation, whereas it did not inhibit

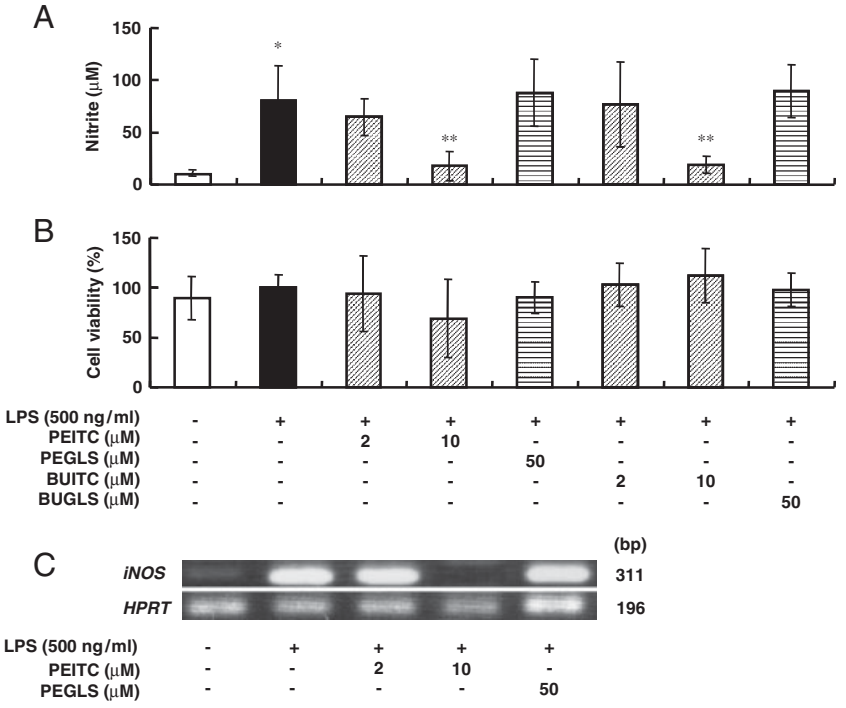

Figure 2. (A, B) PEITC suppressed LPS-induced NO production and *iNOS* mRNA expression. pMϕ (5×10^5 cells/200 μL) were pretreated with or without PEITC, BUITC (2, 10 μM, each), PEGLS, or BUGLS (50 μM) for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL of LPS for 48 h. Nitrite levels in the culture media were determined using Griess assays. Cytotoxicity was evaluated by an MTT test. Each value represents the mean ± SD of triplicate experiments. Student's *t*-test was used to determine significant differences. $^*p<0.05$ *versus* CTL, $^{**}p<0.05$ *versus* LPS. (C) pMϕ (5×10^6 cells/2 mL) were pretreated with PEITC (0, 2, 10 μM) or 50 μM of PEGLS for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL of LPS for 18 h. Total RNA was prepared and RT-PCR was performed. The PCR products of *iNOS* and *HPRT* were separated on agarose gels, and stained with SYBR Gold.

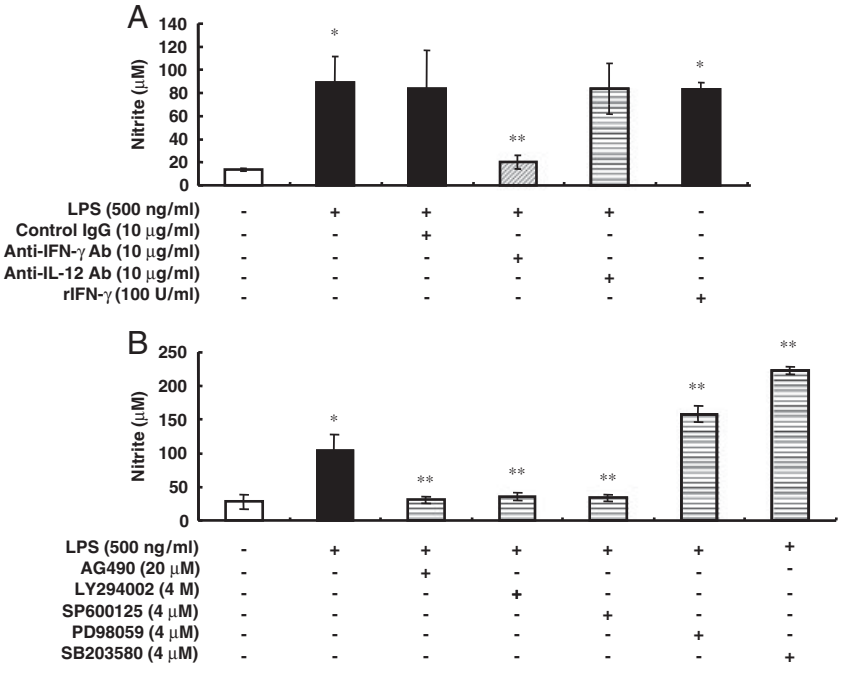

Figure 3. (A) The neutralization antibody of anti-IFN-γ, but not anti-IL-12, suppressed NO production. pMϕ (5×10^5 cells/200 μL) were pretreated with 0.5% DMSO for 30 min, and then exposed to IgG (control), or the neutralization antibody of anti-IFN-γ or that of anti-IL-12 (10 μg/mL, each). (B) pMϕ (5×10^5 cells/200 μL) were pretreated with AG490 (20 μM), LY294002 (4 μM), SP600125 (4 μM), PD98059 (4 μM), or SB203580 (4 μM) for 30 min, and then exposed to 2 mM L-arginine and 500 ng/mL of LPS for 48 h. The concentrations of nitrite in the culture media were determined by Griess assays. Each value represents the mean ± SD of triplicate experiments. Student's *t*-test was used to determine significant differences. $^*p<0.05$ *versus* CTL, $^{**}p<0.05$ *versus* LPS.

LPS-induced phosphorylation of CREB, a transcription factor of IFN-γ [24], at Ser133 (Fig. 5). In addition, PTEN, a negative regulator of the PI3K/Akt pathway, was not activated by any of the experimental treatments. Similarly, PEITC did not suppress LPS-induced IκBα protein degradation, which is the key event for NF-κB activation [25]. Since 6-(methylsulfinyl)hexyl ITC has been reported to suppress iNOS expression through the PI3K/JNK pathway [26], the status of JNK1/2/3 phosphorylation at Thr183/Tyr185 was also evaluated. In contrast to that previous

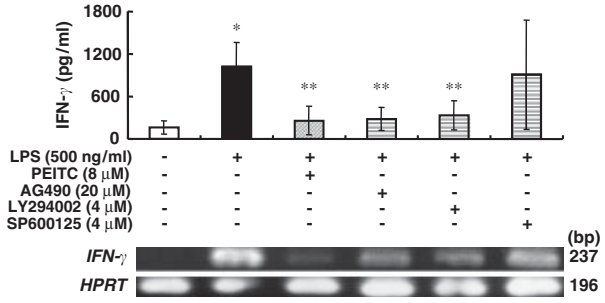

Figure 4. PEITC suppressed LPS-induced IFN-γ secretion and *IFN-γ* mRNA expression. pMϕ (5×10^5 cells/200 μL) were pretreated with PEITC (8 μM), AG490 (20 μM), LY294002 (4 μM), or SP600125 (4 μM) for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL of LPS for 24 h. The concentrations of IFN-γ in the culture media were determined by ELISA. Each value represents the mean ± SD of triplicate experiments. Student's *t*-test was used to determine significant differences. $^*p<0.05$ *versus* CTL, $^{**}p<0.05$ *versus* LPS. pMϕ (5×10^6 cells/2 mL) were pretreated with PEITC (8 μM), AG490 (20 μM), LY294002 (4 μM), or SP600125 (4 μM) for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL of LPS for 6 h. Total RNA was prepared and RT-PCR was performed. The PCR products of *IFN-γ* and *HPRT* were separated on agarose gels, and stained with SYBR Gold.

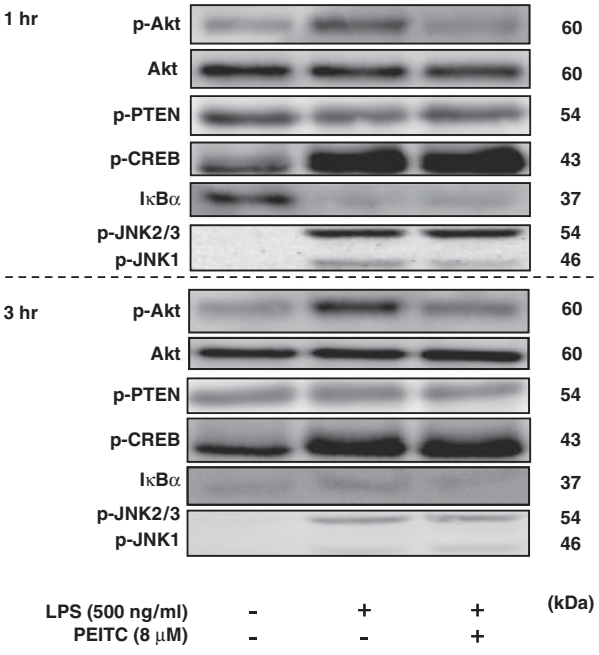

Figure 5. PEITC inhibited the phosphorylation of Akt. pMϕ (5×10^6 cells/2 mL) were pretreated with PEITC (8 μM) for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL of LPS for 1 h. Phospho-Akt, Akt, phospho-PTEN, phospho-CREB, IκBα, and phospho-JNK were detected by Western blot analysis using their specific antibodies.

report, LPS-induced JNK1/2/3 phosphorylation was not inhibited by PEITC. The phosphorylation of Akt, PTEN, CREB, JNK1/2/3 at 3 h after LPS stimulation and the effects of PEITC on them were almost similar to those at 1 h (Fig. 5).

3.6 PEITC suppresses activation of JAK2/STAT1 signaling pathway

It is well known that IFN-γ activates the JAK2/STAT1 signaling pathway, thereby inducing iNOS expression [27]. We assessed JAK2/STAT1 phosphorylation at 1 and 3 h after LPS stimulation (Fig. 6A). Both JAK2 and STAT1 were phosphorylated at 3 h, but not 1 h, after LPS stimulation, and PEITC abolished the phosphorylation of both. In addition, PEITC was found to attenuate IFN-γ-induced JAK2 phosphorylation and, to a lesser extent, that of STAT1 (Fig. 6B).

3.7 PEITC suppresses NOx production and IFN-γ secretion in mice

We also investigated the effects of PEITC and its related compounds on NO production *in vivo*. ICR mice were given PEITC, PEGLS, BUITC, or BUGLS (each 7.5 mmol/kg in the diet) from the start of the experiment for 44 h and injected intraperitoneally with LPS (1 mg/kg of body weight) at 24 h after starting the experiment, after which sera were collected at 20 h after LPS injection. As shown in Fig. 7A, LPS increased the concentration of NOx by 18-fold, whereas PEITC, but not PEGLS, suppressed it by 64% ($p<0.05$). In addition, both BUITC and BUGLS showed a tendency to suppress that concentration, though it was not statistically significant. Similar results were obtained when we measured the concentrations of IFN-γ in the sera (Fig. 7B).

4 Discussion

IFN-γ, IL-12, and IL-18 have been reported to mediate NO production in LPS-activated peritoneal exudates cells (PECs) from C3H/HeN strain mice [8]. Furthermore, that study indicated that PECs generally contain 87–92% Mϕ and 6–9% lymphocytes, and that IL-12 and IL-18 from Mϕ act synergistically on Th1 and NK cells to secrete IFN-γ, which stimulates Mϕ to produce NO. In fact, when non-adherent cells were washed from the PECs in their experiments, the remaining adherent cells were mostly Mϕ, which showed a remarkable reduction of IFN-γ secretion in response to LPS stimulation. This mechanism corresponds to the classic model, in which IL-12 from Mϕ acts on Th1 or NK cells to produce IFN-γ [28]. However, in this study, IL-12 did not mediate NO production in LPS-activated pMϕ from the ICR mice (Fig. 3A). This discrepancy may be due to differences between the mouse strains. In fact, pMϕ from C3H/OuJ mice are induced by LPS and increase the steady-state level of *IFN-γ* mRNA, while those from C3H/HeJ mice are not

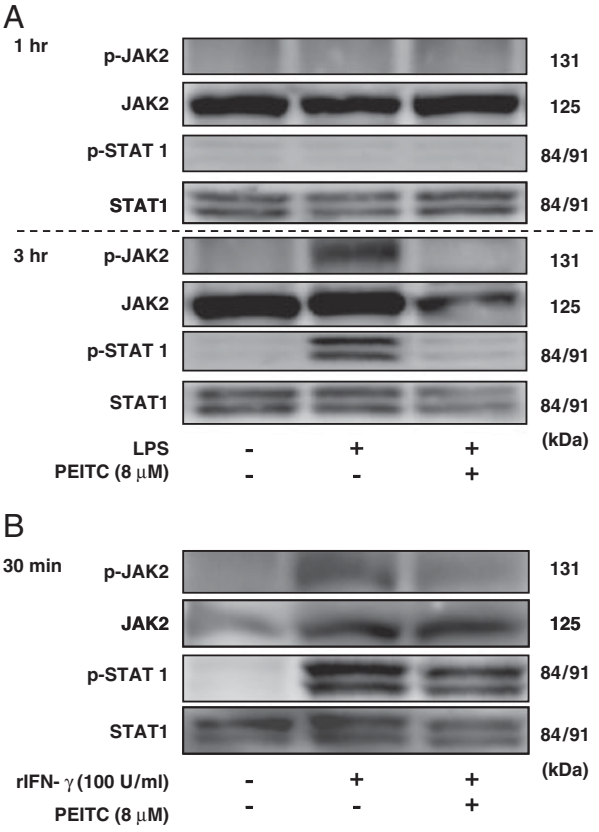

Figure 6. PEITC suppressed the phosphorylation of JAK2 and STAT1 induced by (A) LPS and (B) IFN-γ. (A) pMϕ (5×10^6 cells/2 mL) were pretreated with PEITC (8 μM) for 30 min, and then exposed to 2 mM of L-arginine and 500 ng/mL LPS for 1 and 3 h. Phospho-JAK2 and JAK2, and phospho-STAT1 and STAT1 were detected by Western blot analysis using their specific antibodies. (B) pMϕ (5×10^6 cells/2 mL) were pretreated with PEITC (8 μM) for 30 min, and then exposed to 2 mM of L-arginine and 100 U/mL of recombinant IFN-γ for 30 min. Phospho-JAK2 and JAK2, and phospho-STAT1 and STAT1 were detected by Western blot analysis using their specific antibodies.

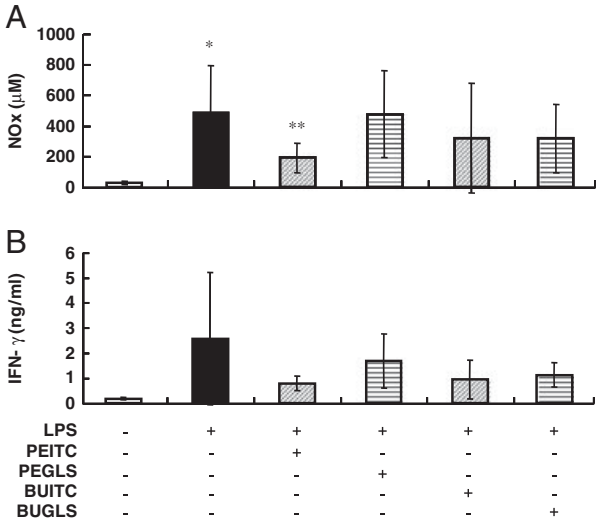

Figure 7. ICR mice were given PEITC, PEGLS, BUITC, or BUGLS (7.5 mmol/kg in the diet) ($n = 4$ in each group) from the start of the experiment for 44 h. Next, the mice were injected with LPS (1 mg/kg body weight) intraperitoneally at 24 h after starting the experiment and blood samples were collected at 20 h after LPS injection. NOx and IFN-γ concentrations in sera were determined using an NOx analyzing system and ELISA, respectively. The experiment was performed twice. Each value represents the mean ± SD and Student's *t*-test was used to determine significant differences. $^*p < 0.05$ *versus* CTL, $^{**}p < 0.05$ *versus* LPS.

induced by LPS [29]. Moreover, IFN-γ is induced in Mϕ from BALB/c mice in response to *Legionella pneumophila*, whereas IFN-γ is not induced in those from A/J mice [30]. In addition, splenocytes secrete IFN-γ in an IL-12-independent manner upon stimulation by Gram-negative bacteria [31]. In this study, we found that IFN-γ, but not IL-12, is released by LPS in pMϕ and probably mediates NO production (Fig. 3A). The molecular mechanism by which LPS induces *IFN-γ* mRNA expression should be addressed in the future.

PEITC inhibited phosphorylation of Akt at Ser473, but did not activate PTEN (Fig. 5), suggesting that PEITC acts on Akt or upstream signaling molecule(s) other than PTEN. The PI3K/Akt signaling pathway plays an important role in LPS-induced NO production [11]. p85, a regulatory subunit of PI3K, is activated *via* binding to myeloid differentiation factor 88, an adaptor protein of TLR4, after recognition of LPS by TLR4 [12]. Moreover, in human monocytes, CD-14-associated Lyn, one of the STKs, is activated by LPS [32], then interacts with p85 and activates PI3K [33]. In this study, AG490 (20 μM) inhibited LPS-induced IFN-γ mRNA expression and secretion (Fig. 4), suggesting that JAK2 may be associated with PI3 K activation. In support of this notion, Okugawa *et al.* reported that JAK2 is involved in PI3K activation in LPS-challenged RAW264.7 cells [23].

Although CREB, AP-1, and NF-κB are transcription factors related to the *IFN-γ* gene [24, 34, 35], PEITC showed no effects on them (Fig. 5). On the other hand, the effects of PEITC on NF of activated T cells [36], STAT4 [37], and T-bet [38] remain to be addressed in the future. In RAW264.7 Mϕ, PEITC was previously shown to suppress iNOS expression by inhibiting IκBα degradation in response to LPS stimulation [20]. However, PEITC did not inhibit LPS-induced IκBα degradation in pMϕ in this study (Fig. 5). This discrepancy may be due to the different experimental conditions utilized, including the cell lines.

Although PEITC suppressed LPS-induced JAK2/STAT1 activation (Fig. 6A), it is unclear if it can be attributable to IFN-γ suppression or independently acts on the JAK2/STAT1 pathway. We investigated the effects of PEITC on IFN-γ-induced phosphorylation of JAK2/STAT1 and found marked suppression (Fig. 6B). Collectively, inactivation of the JAK2/STAT1 pathway by PEITC may be due to down-regulation of both IFN-γ secretion and the IFN-γ signaling pathway, which involves JAK2/STAT1. Lin *et al.* docu-

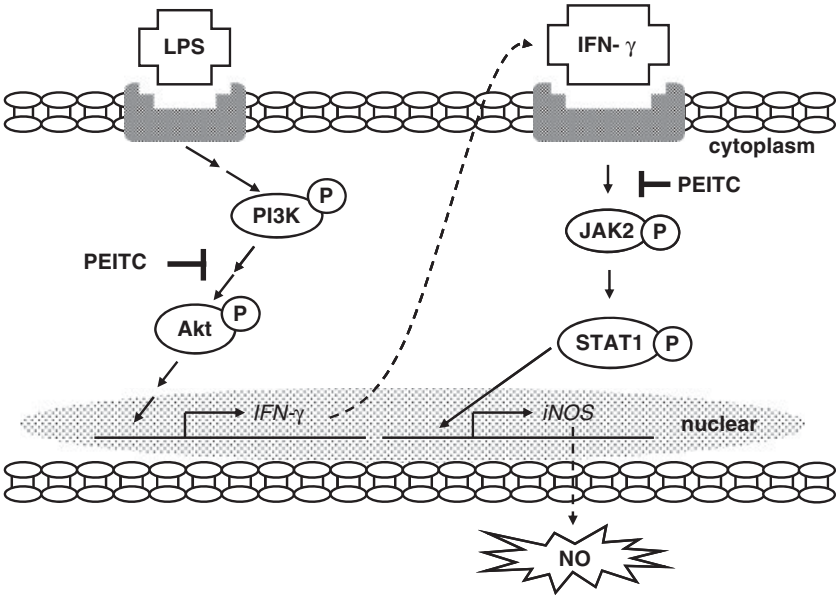

Figure 8. Proposed schema of the molecular mechanisms by which PEITC suppresses NO production in pMϕ.

mented that sulforaphane, one of the major ITCs in nature, suppressed LPS-induced expression of inflammatory genes including *iNOS via* an NF-E2-related factor 2-dependent pathway in pMϕ [39]. On the other hand, glutathione-*S*-transferase P1 (GSTP1), which plays an important role in the detoxification system, inhibited LPS-induced MAPKs, including extracellular signal-regulated kinase, p38 MAPK, and JNK, as well as NF-κB activation, and thereby decreased NO production in RAW264.7 cells [40]. Furthermore, GSTP1 directly interacts with the *C*-terminal of JNK, and functions as an endogenous inhibitor of JNK [41] and with TRAF2 to inhibit TNF-α-induced signaling [42]. Interestingly, extracellular recombinant GSTP1 was found to suppress the expression of *iNOS* in both *in vitro* and *in vivo* conditions [43]. In addition, it is well known that many isothiocyanates, including PEITC, activate NF-E2-related factor 2 [44] and induce GSTP1 [45], and that MAPK participates in the PI3K/Akt pathway of LPS signaling in RAW264.7 Mϕ [46]. Thus, putative induction of GSTP1 by PEITC may be associated with regulation of signaling molecules upstream of Akt.

Cruciferous vegetables are thought to have protective effects against various cancers and epidemiological surveys have implied that the consumption of cruciferous vegetables, including cabbage, kale, broccoli, brussel sprouts, and cauliflower, decrease the risk of lung, stomach, colon, and rectal cancers [47]. Meanwhile, it has been long suggested that chronic inflammation is a risk factor for carcinogenesis [48]. To the best of our knowledge, this study is one of the first to find that PEITC is orally active for suppressing LPS-induced NO production in inflammation model mice. Our results showed that the suppressive tendency of PEITC and its related compounds on NOx production and IFN-γ secretion were similar (Fig. 7), suggesting that IFN-γ mediates NO production in LPS-stimulated ICR mice. Intriguingly, PEGLS did not suppress NOx production, but tended to suppress IFN-γ secretion (Fig. 7B). This may be because bacteria with myrosinase activity in the bowel microflora catalyze the hydrolysis of glucosinolates for generating isothiocyanates [49]. Along a similar line, it is of note that BUGLS showed an anti-inflammatory effect similar to that of BUITC (Figs. 7A and B).

In conclusion, our results suggest that PEITC suppresses LPS-induced NO production in pMϕ *via* inhibition of Akt activation and the resultant decrease of secretion of IFN-γ, a critical autocrine mediator of NO production. Additionally, PEITC was revealed to be a potent suppressor of the JAK2/STAT1 signaling pathway (Fig. 8).

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