In vitro anti-inflammatory effects of the phenylbutyric acid metabolite phenylacetyl glutamine

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Summary

Sodium 4-phenylbutyrate (PBA), which exerts a wide range of anti-inflammatory effects, is rapidly cleared from the body (approximately 98%) by urinary excretion by 24 h after oral treatment in humans. PBA was almost entirely excreted to urine as phenylacetyl glutamine (PAGln). However, no data describe the potential anti-inflammatory effects of PAGln. The purpose of this study was to evaluate the anti-inflammatory effects of PAGln on mouse spleen cells and peritoneal cavity cells, and explore the potential mechanism underlying this effect.

PAGln was added to mouse spleen cell cultures stimulated by concanavalin A, or mouse peritoneal cavity cell cultures stimulated by lipopolysaccharide. After 72 h of culture, levels of inflammatory cytokines in culture supernatants were measured using a sandwich enzyme-linked immunosorbent assay system, and levels of inflammatory proteins were assessed by western blotting.

PAGln significantly inhibited inflammatory cytokine (interferon-γ, interleukin-6, and tumor necrosis factor-α) production, decrease of cell number in the spleen cell, and suppressed the expression of inflammatory proteins (nuclear factor κB, and inducible nitric oxide synthase). These results suggest that PAGln possesses anti-inflammatory activity via inhibition of T cell activation and Toll-like receptor 4 signaling. This study of the anti-inflammatory mechanism of PAGln provides useful information about its potential for therapeutic applications.

Key words
sodium 4-phenylbutyrate, phenylacetyl glutamine, cytokines, spleen cell, peritoneal cavity cell, Toll-like receptor 4
1. **Introduction**

Sodium 4-phenylbutyrate (PBA) is a phenyl-substituted short-chain fatty acid used in the treatment of a wide range of diseases, such as urea cycle disorders\(^1\), homozygous β-thalassemia\(^2\), spinal muscular atrophy\(^3\), and tumors\(^4\). Molecular activities of PBA include ammonia scavenging, chaperoning, and inhibition of histone deacetylases\(^5\).

PBA reportedly suppresses oxidative stress by attenuating endoplasmic reticulum stress, which results in the anti-inflammatory effect of inhibiting nuclear factor-κB (NF-κB) activity\(^6\). We previously reported that PBA may be effective for the treatment of neurodegenerative diseases, including Parkinson’s disease\(^7\). Furthermore, PBA was reported to show anti-inflammatory effects in a mouse model of colitis induced by dextran sulfate sodium, a standard animal model of inflammatory bowel disease utilized in our previous study\(^8,9\).

In spite of the reported multiple effects of PBA, its usefulness as a therapeutic agent in humans has been limited by its short half-life, rapid metabolism, and fast excretion *in vivo*\(^10\). One study reported that PBA was rapidly cleared from the human body (approximately 98%) by urinary excretion 24 h after oral treatment. As shown in Figure 1, PBA was excreted to urine as 93% phenylacetyl glutamine (PAGln), 6% phenylacetyl taurine (PAT), and 0.05% phenylacetyl glycine (PAGly)\(^11\). While it is possible that the PAGln metabolite of PBA exerted anti-inflammatory effects *in vivo* as a secondary action, rather than PBA directly, no previously reports describe an anti-inflammatory effect of PAGln.

In this study, we first examined the anti-inflammatory effect of PAGln *in vitro*. The results provide useful information for potential future therapeutic applications of PAGln.
2. Materials and methods

2.1 Animals
ICR mice (male, 5 weeks of age) weighing 18–22 g were purchased from the Kyudo Laboratory Animal Center (Fukuoka, Japan) and housed in polypropylene cages with sawdust bedding. The temperature was maintained at 24 ± 1°C, with a humidity of 50 ± 10% and 12-h/12-h light/dark cycles. Food and water were available ad libitum. Procedures used for animals and their care followed internationally accepted Guidelines for Keeping Experimental Animals, issued by the Government of Japan. The researchers received ethical training from the Fukuoka University Ethics Committee.

2.2 PAGln and Gln treatment
PAGln and Gln were supplied by Sigma-Aldrich (MO, USA). PAGln and Gln were diluted with distilled water to three concentrations. The solution was sterilized by filtration through a 0.22-µM membrane. PAGln and Gln were added to culture medium in cell culture plates.

2.3 Cell culture

2.3.1 Spleen cell proliferation assay
Spleen cells were prepared as described in a previous study\textsuperscript{12}). Spleen cells were seeded in a 96-well plate (Nunc) at a density of 5.0 × 10\textsuperscript{6} cells/mL. Subsequently, concanavalin A (Con-A, 5 µg/mL in medium; Sigma-Aldrich, St. Louis, MO, USA) was added, plates were incubated for 72 h at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. Subsequently, spleen cell proliferation was determined using a WST-8 assay kit (Nacalai Tesque, Kyoto, Japan), according to the manual. The absorbance was determined at 450 nm using a microplate reader.
2.3.2 Peritoneal cavity cell proliferation assay

Peritoneal macrophages isolated from male ICR mice (5 weeks-old). The collected cells from mice were seeded in 96-well flat-bottom plates (Nunc) at a density of $1.0 \times 10^6$ cells/ml. Subsequently, lipopolysaccharide (LPS, 10 µg/mL in medium; Sigma-Aldrich, St. Louis, MO, USA) was added, plates were incubated for 72 h at 37°C in a humidified atmosphere with 5% CO₂.

2.4 Measurement of cytokines using enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in sterilized supernatants of Con-A–stimulated spleen cell or LPS-stimulated peritoneal cavity cells were measured at 72 h using ELISA. Absorbance was measured at 450 nm with a Bio-Rad Model 680 Microplate Reader (Bio-Rad, Hercules, CA). All anti-cytokine antibodies were purchased from eBioscience (San Diego, CA).

2.5 Immunoblot analysis

For detection of inflammatory proteins, cells were lysed in RIPA buffer. Total protein concentration was measured using a BCA assay (Pierce Biotechnology, Rockford, IL). Cell lysates (containing equal amounts of protein) were subjected to SDS-PAGE (Bio-Rad) and proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with Blocking One overnight at 4°C, and were then incubated for 1 h at RT with anti-iNOS (induced nitric oxide synthase), anti-NF-κB, anti-phosphorylated NF-κB (Cell Signaling Technology, Beverly, MA), or anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibody at a 1:500 dilution in blocking solution. After washing three times, membranes were incubated for 1 h at RT with an appropriate horseradish peroxidase-conjugated species-specific secondary antibody. Immunoreactive bands were visualized with ImmunoStar® LD (Wako Pure Chemical Industries, Osaka, Japan).
2.6 Statistical analysis

Results are expressed as mean ± SD (n = 3). Data were evaluated for statistical significance using the Bonferroni test for differences between groups. Overall significance was determined using a one-way ANOVA (repeated measures). A $p$-value of < 0.05 was considered to be statistically significant.
3. Results

3.1 Effect of PAGln or Gln on inflammatory cytokine secretion from Con-A–stimulated spleen cells

Levels of IFN-γ, IL-6, and TNF-α production were increased in mouse spleen cells stimulated by Con-A. This increased production was significantly suppressed by the addition of PAGln in a dose-dependent manner, as shown in Figure 2. Levels of IFN-γ and TNF-α production were also suppressed by the addition of Gln, as shown in Figure 2A and 2C.

3.2 Effect of PAGln or Gln on cell viability

Spleen cells stimulated by Con-A or peritoneal cavity cells stimulated by LPS were treated with different doses of PAGln and Gln. Cell survival was evaluated in WST-8 assay. As shown in Table 1, after 72 h of incubation and stimulation with Con-A, PAGln suppressed both cell viability and proliferation of spleen cells in a dose-dependent manner. However, there was no significant change in LPS-stimulated peritoneal cavity cells treated with PAGln or Gln.

3.3 Inhibitory effect of PAGln and Gln on NF-κB, phosphorylated NF-κB and iNOS in Con-A–stimulated mouse spleen cells

As shown in Figure 3A and 3B, expression levels of NF-κB in Con-A–stimulated mouse spleen cells were significantly inhibited by PAGln in a dose-dependent manner. However, there was no significant change in the phosphorylation of NF-κB (p-NF-κB) as showed in Figure 3C. Furthermore, iNOS expression levels were also significantly inhibited by PAGln these cells, as shown in Figure 4.
3.4 Effect of PAGln or Gln on inflammatory cytokine secretion from LPS-stimulated peritoneal cavity cells

Levels of IL-6 and TNF-α production were increased in LPS-stimulated peritoneal cavity cells in a dose-dependent manner, as shown in Figure 5. However, levels of IFN-γ levels secretion were not increased by LPS stimulation of peritoneal cavity cells.

3.5 Inhibitory effect of PAGln or Gln on iNOS expression in LPS-stimulated peritoneal cavity cells

As shown in Figure 6, iNOS expression levels in LPS-stimulated peritoneal cavity cells were significantly inhibited by PAGln in a dose-dependent manner.

4. Discussion

The anti-inflammatory mechanism of PAGln was investigated in this study using spleen cells stimulated with Con-A, a T cell activator, and peritoneal cavity cells stimulated with LPS, a TLR4 agonist.

As shown in Figures 2–4, levels of IFN-γ, IL-6, and TNF-α were significantly suppressed with PAGln treatment. It was possible that the suppression of cytokines were due to decrease of number of spleen cells as shown in Table 1. Furthermore, expression of NF-κB and iNOS were significantly suppressed by PAGln treatment. These results were correlated with previous data for PBA6. However, we observed no significant differences in the ratio of p-NF-κB/NF-κB, suggesting that PAGln suppressed the level of total NF-κB, but not NF-κB phosphorylation. In addition, levels of IFN-γ and TNF-α production were also suppressed by the addition of Gln, as shown in Figure 2A and 2C. Our data for Gln correlated with previous reports of Gln exerting a suppressive effect on cytokine stimulation13) and inhibiting I-κB kinase β/NF-κB activation14). However, the anti-inflammatory effects of PAGln were greater
than observed for Gln. These results indicated that PAGln exerted significant anti-inflammatory effects.

As shown in Figures 5 and 6, levels of IL-6 and TNF-α were significantly suppressed by PAGln treatment in supernatants from peritoneal cavity cells without decrease of cell number as shown in Table 1. Furthermore, iNOS expression levels were significantly suppressed by treatment with PAGln. These results suggest that PAGln inhibited peritoneal cavity cell activation stimulated by a TLR4 agonist. Thus PAGln affected for T cell activation and peritoneal cavity activation by a TLR4 agonist in different mechanism.

While, the expression of NF-κB and P-NF-κB in LPS-stimulated peritoneal cavity cells also examined (data not shown). However, the expression of NF-κB and P-NF-κB were disappeared when peritoneal cavity cells were stimulated with LPS though NF-κB was expressed in peritoneal cavity cells without LPS. In this peritoneal cavity cell culture, the levels of NF-κB or P- NF-κB could not be evaluated when PAGln was added to peritoneal cavity cells stimulated by LPS. It has been reported that estrogen-related receptor α (ERRα) negatively regulated TLR4-induced inflammatory response by regulation of NF-κB signaling in macrophages in mice\(^\text{15}\). Furthermore, the expression of ERRα was strongly upregulated by stimulation with TLR agonist in its previous report\(^\text{15}\). From these reports, there were possible that LPS as a TLR4 agonist upregulated ERRα expression, NF-κB levels were reduced in peritoneal cavity cells stimulated by LPS in this study.

It has been reported that PBA suppressed the level of IL-6, TNF-α in LPS-induced mouse model of acute lung injury\(^\text{16}\). PBA also inhibited iNOS, NF-κB activation in Experimental allergic encephalomyelitis model\(^\text{17}\). Our results of PAGln were correlated with these previous studies about PBA. That is why, these results suggested that it is possible PAGln might be bioactive body of PBA in vivo because PBA is short half-life.

In interview form of PBA\(^\text{11}\), the C\(_{\text{max}}\) of PBA was 0.5-0.7 mM, the one of PAGln was
0.1-0.2 mM, when PBA were administrated by oral in human. In previous study, PBA showed anti-inflammatory effects at 1, 2, 5 mM in vitro. In this study, PAGln was used at 0.03, 0.6, 10 mM. From our results, it is possible that PAGln might be used at lower dose compared with PBA.

**Conclusion**

In this study, PAGln exerted anti-inflammatory effects in Con-A stimulated spleen cell and LPS-stimulated peritoneal cavity cell. Indeed, our investigation of the anti-inflammatory mechanism of PAGln has provided useful information about its potential therapeutic applications.

5. **Acknowledgments**

We thank Amy Van Deusen from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

6. **Conflict of interest**

The authors declare no conflict of interest.
References


11) Buphenyl® Interview Form, OrphanPacific, Inc. in Japan, 2015.


Table 1. Effect of PAGln or Gln on cell viability in spleen cells and peritoneal cavity cells.

Spleen cells stimulated by Con-A or peritoneal cavity cells stimulated by LPS were treated with PAGln or Gln at 0.03, 0.06, or 10 mM for 72 h. Data are presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01 vs. Control group without PAGln or Gln (Bonferroni test/ANOVA).

Figure 1. Metabolic pathway of PBA in humans.

Figure 2. Effect of PAGln or Gln on secretion of IFN-γ, IL-6, and TNF-α from Con-A–stimulated spleen cells.

Cytokine levels of IFN-γ (A), IL-6 (B), and TNF-α (C) in the supernatant of mouse spleen cell cultures treated with PAGln or Gln at 0.03, 0.06, or 10 mM and Con-A for 72 h were analyzed by ELISA. Data are presented as mean ± SD (n = 3). ###p < 0.01 vs. Con-A(-) group, *p < 0.05, **p < 0.01 vs. Con-A(+) without PAGln or Gln group (Bonferroni test/ANOVA).

Figure 3. Effect of PAGln or Gln on NF-κB expression and phosphorylation in Con-A–stimulated spleen cells.

Spleen cells were treated with PAGln or Gln, and stimulated with Con-A for 72 h. Representative images of western blots showing expression of p-NF-κB and NF-κB (A).

The ratio of NF-κB/β-actin (B) and p-NF-κB/NF-κB (C) were exhibited. Data are presented as mean ± SD (n = 3). ###p < 0.01 vs. Con-A(-) group, * p < 0.05, **p < 0.01 vs. Con-A(+) without PAGln or Gln group (Bonferroni test/ANOVA).
Figure 4. Effect of PAGln or Gln on iNOS expression levels in Con-A–stimulated spleen cells.

Spleen cells were treated with PAGln or Gln, and stimulated with Con-A for 72 h. A representative image of a western blot showing iNOS expression levels is shown. Data are presented as mean ± SD (n = 3). ## $p < 0.01$ vs. Con-A(-) group, **$p < 0.01$ vs. Con-A(+) without PAGln or Gln group (Bonferroni test/ANOVA).

Figure 5. Effect of PAGln or Gln on secretion of IFN-$\gamma$, IL-6, and TNF-$\alpha$ by LPS-stimulated peritoneal cavity cells.

Levels of cytokines IL-6 (A) and TNF-$\alpha$ (B) in the supernatant of mouse peritoneal cavity cell cultures treated with PAGln or Gln at 0.03, 0.06, or 10 mM, and LPS for 72 h were analyzed by ELISA. Data are presented as mean ± SD (n = 3). ## $p < 0.01$ vs. LPS(-) group, *$p < 0.05$, **$p < 0.01$ vs. LPS(+) without PAGln or Gln group (Bonferroni test/ANOVA).

Figure 6. Effect of PAGln or Gln on iNOS expression levels in LPS-stimulated peritoneal cavity cells.

Peritoneal cavity cells were treated with PAGln or Gln, and stimulated with LPS for 72 h. A representative image of a western blot showing iNOS expression is shown. Data are presented as mean ± SD (n = 3). ## $p < 0.01$ vs. LPS(-) group, **$p < 0.01$ vs. LPS(+) without PAGln or Gln group (Bonferroni test/ANOVA).
Table 1

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<td>Peritoneal cavity cell</td>
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<td>Gln 10 mM</td>
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sodium 4-phenylbutyrate (PBA)

β-oxidation

phenylacetate

phenylacetyl glutamine (PAGln)
(urea: 93%)

phenylacetyl taurine (PAT)
(urea: 6%)

phenylacetyl glycine (PAGly)
(urea: <0.05%)
Figure 2

(A) IFN-γ
(B) IL-6
(C) TNF-α

Con-A(-) Con-A(+)

Con-A(-) Con-A(+)

Con-A(-) Con-A(+)

** ## ##

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Figure 3

(A) PAGln Gln PAGln Gln

(B) Con-A(-) Con-A (+)

(C) Con-A(-) Con-A (+)

P-NF-κB/NF-κB 65 kDa

β-actin 42 kDa

Con-A (+)

Con-A (-)

0 0 0.03 0.6 10 0.03 0.6 10 (mM)

NF-κB 65 kDa

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Figure 5

(A) IL-6 (pg/ml)

(B) TNF-α (pg/ml)

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**Biological and Pharmaceutical Bulletin Advance Publication**
Figure 6

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