Anti-inflammatory effect of pyroglutamyl-leucine on lipopolysaccharide-stimulated RAW 264.7 macrophages

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A B S T R A C T
Aims: Food-derived peptides have been reported to yield a variety of health promoting activities. Pyroglutamyl peptides are contained in the wheat gluten hydrolysate. In the present study, we investigated the effect of pyroglutamyl dipeptides on the lipopolysaccharide (LPS)-induced inflammation in macrophages.

Main methods: RAW 264.7 macrophages were treated with LPS and various concentrations of pyroglutamyl-leucine (pyroGlu-Leu), -valine (pyroGlu-Val), -methionine (pyroGlu-Met), and -phenylalanine (pyroGlu-Phe). Cell viability/proliferation and various inflammatory parameters were measured by the established methods including ELISA and western blotting. The binding of fluorescein isothiocyanate-labeled LPS to RAW 264.7 cells was also measured fluorescently.

Key findings: All the tested dipeptides significantly inhibited the secretion of nitric oxide, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 from LPS-stimulated RAW 264.7 macrophages. Above all, pyroGlu-Leu inhibited the secretion of all these inflammatory mediators even at the lowest dose (200 μg/ml). PyroGlu-Leu dose-dependently suppressed IκB degradation and MAPK (JNK, ERK, and p38) phosphorylation in LPS-stimulated RAW 264.7 cells. On the other hand, it did not affect the binding of LPS to the cell surface.

Significance: Our results indicated that pyroGlu-Leu inhibits LPS-induced inflammatory response via the blocking of NF-κB and MAPK pathways in RAW 264.7 macrophages.

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Introduction

Inflammation is a host response to tissue injury and infections that leads to the release of a large amount of inflammatory mediators. Over-activated or chronic inflammation contributes to the pathogenesis of many diseases such as bronchitis (Vernooy et al., 2002), chronic renal disease (Sean Eardley and Cockwell, 2005), and cancer in various tissues (Macciò and Madeddu, 2012; Santiago et al., 2007; Szabo et al., 2010). Macrophages play a critical role in the process of inflammation in many different tissues (Martinez et al., 2009). There are several pro-inflammatory mediators involved during an inflammatory response. Lipopolysaccharide (LPS), which is one of the most potent initiators of inflammation derived from the outer membrane of gram negative bacteria, activates several signaling pathways including inhibitory κB (IκB)/nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) by acting on toll-like receptor (TLR) 4 to induce the expression of inflammatory genes and the release of mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Guha and Mackman, 2001). NO is a free oxygen radical and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Alderton et al., 2001; Bogdan, 2001). NO is produced from l-arginine by inducible nitric oxide synthase (iNOS) during inflammation. TNF-α and IL-6 are pivotal pro-inflammatory cytokines which are involved in a variety of immune responses leading to inflammation (Beutler and Cerami, 1989; Delgado et al., 2003).

In the past several decades, many researchers have reported that food-derived peptides yield a variety of health promoting activities, including a reduction of blood pressure (Karaki et al., 1993; Sipola et al., 2001), modulation of immune cell functions (Lahov and Regelson, 1996; Miyachi et al., 1997), and regulation of nerve functions (Yang et al., 2001; Yoshikawa et al., 1984). Wheat gluten has a unique amino acid composition: glutamyl residues account for about 40% of the amino acids (Kasarda et al., 1984). It is demonstrated that wheat gluten hydrolysate (WGH) contains various functional peptides which have opioidergic activity (Morley et al., 1983; Schusdziarra et al., 1981) or inhibitory effect on angiotensin-I converting enzyme (Motoi and Kodama, 2003). More-
recently demonstrated that pyroglutamyl-leucine (pyroGlu-Leu) is the active component in WGH that exerts hepatoprotective activity. These results suggest that pyroglutamyl peptides, especially pyroGlu-Leu, may be the effective components that act on these inflammatory diseases; however, the underlying mechanism has not been clarified.

In the present study, we examined the effect of some pyroglutamyl dipeptides (Fig. 1) on inflammatory responses in vitro, and determined the underlying mechanism of the action of pyroglutamyl-leucine (pyroGlu-Leu) in LPS-stimulated RAW 264.7 macrophages.

Materials and methods

Peptide synthesis

All the dipeptides were synthesized by a conventional solution method as described below. Reagents and solvents were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and used without further purification. All the amino acid derivatives for peptide synthesis were purchased from Watanabe Chemical (Hiroshima, Japan). Reagents for peptide synthesis, N-hydroxybenzotriazole (HOBT), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide monohydrochloride (EDC·HCl), were purchased from Peptide Institute Inc. (Osaka, Japan). The final products were analyzed by reversed-phase HPLC using a Shimazu high-pressure gradient system consisting of two LC-10ADvp pumps and a SPD-10Avp UV–Vis detector and a Tosoh analytical C18 column (TSKgel ODS-100S, 3.0 × 150 mm). Molecular ion masses of the synthesized peptides were determined by electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) on a Hitachi NanoFrontier LC–MS system (Tokyo, Japan).

Pyroglutamyl-leucine (pyroGlu-Leu)

To a solution of HCl·Leu-OtBu (500 mg, 2.2 mmol) in N,N-dimethylformamide (DMF) (15 ml) was added triethylamine (Et3N) (0.31 ml, 2.2 mmol) and Boc-pyroGlu-OH (510 mg, 2.2 mol) at 0 °C. HOBT (600 mg, 4.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide monohydrochloride (EDC·HCl) (640 mg, 3.3 mmol) were added and the reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, and washed with 5% NaHCO3 and 10% citric acid, and dried over anhydrous sodium sulfate. After filtration, the resulting filtrate was concentrated under reduced pressure and white product was precipitated by petroleum ether to give 850 mg of protected dipeptide in 96% yield. The protected dipeptide was treated with 4 M HCl/dioxane at room temperature for 3 h to remove the protected groups. After the removal of excess HCl/dioxane, white solid was precipitated by diethyl ether to give 430 mg of desired product in 85% yield. MS (ESI) m/z: 243.09 [M + H]+ (calcd. 243.13).

Pyroglutamyl-valine (pyroGlu-Val)

To a solution of HCl·Val-OtBu (210 mg, 1.0 mmol) in DMF (10 ml) was added Et3N (0.15 ml, 1.1 mmol) and Boc-pyroGlu-OH (229 mg, 1.0 mol) at 0 °C. HOBT (271 mg, 2.0 mmol) and EDC·HCl (287 mg, 1.5 mmol) were added and the reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, and washed with 5% NaHCO3, and 10% citric acid, and dried over anhydrous sodium sulfate. After filtration, the resulting filtrate was concentrated under reduced pressure and white product was precipitated by petroleum ether to give 325 mg of protected dipeptide in 85% yield. The protected dipeptide was treated with 4 M HCl/dioxane at room temperature for 3 h to remove the protected groups. After the removal of excess HCl/dioxane, white solid was precipitated by diethyl ether to give 202 mg of desired product in 85% yield. MS (ESI) m/z: 229.13 [M + H]+ (calcd. 229.11).

Pyroglutamyl-methionine (pyroGlu-Met)

To a solution of HCl·Met-OtBu (242 mg, 1.0 mmol) in DMF (10 ml) was added Et3N (0.15 ml, 1.1 mmol) and Boc-pyroGlu-OH (229 mg, 1.0 mol) at 0 °C. HOBT (271 mg, 2.0 mmol) and EDC·HCl (287 mg, 1.5 mmol) were added and the reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, and washed with 5% NaHCO3 and 10% citric acid, and dried over anhydrous sodium sulfate. After filtration, the resulting filtrate was concentrated under reduced pressure and white product was precipitated by petroleum ether to give 248 mg of protected dipeptide in 59% yield. The protected dipeptide (198 mg) was treated with 4 M HCl/dioxane at room temperature for 3 h to remove the protected groups. After the removal of excess HCl/dioxane, white solid was precipitated by diethyl ether to give 90 mg of desired product in 73% yield. MS (ESI) m/z: 261.09 [M + H]+ (calcd. 261.09).

Fig. 1. Structures of pyroglutamyl-leucine (A), pyroglutamyl-valine (B), pyroglutamyl-methionine (C), and pyroglutamyl-phenylalanine (D).
Pyroglutamyl-phenylalanine (pyroGlu-Phe)

To a solution of HCl-Phe-ObBu (1.29 g, 5.0 mmol) in DMF (50 ml) was added Et3N (0.77 ml, 5.5 mmol) and Boc-pyroGlu-OH (1.15 g, 5.0 mol) at 0 °C. HOBr (1.35 g, 10 mmol) and EDC-HCl (1.44 g, 7.5 mmol) were added and the reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, and washed with 5% NaHCO3 and 10% citric acid, and dried over anhydrous sodium sulfate. After filtration, the resulting filtrate was concentrated under reduced pressure and white product was precipitated by petroleum ether to give 2.03 g of protected dipeptide in 94% yield. The protected dipeptide (502 mg, 1.2 mmol) was treated with 4 M HCl/dioxane at room temperature for 3 h to remove the protected groups. After the removal of excess HCl/dioxane, white solid was precipitated by ether to give 285 mg of desired product in 89% yield. MS (ESI) m/z: 277.13 [M + H]+ (calcd. 277.11).

Cell culture

RAW 264.7 macrophages (RIKEN BioResource Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco BRL, NY, USA) at 37 °C in a humidified 5% CO2 atmosphere. The cells were seeded on 24 well-plates (5 x 105 cells/ml) and treated with 2 μg/ml LPS (Sigma, MO, USA) and various concentrations of pyroglutamyl dipeptides (pyroGlu-Leu, pyroGlu-Val, pyroGlu-Met, and pyroGlu-Phe) in a serum free medium.

Cell viability assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, WI, USA) was used to evaluate the cytotoxicity of each dipeptide. RAW 264.7 macrophages were seeded in 96-well plates (5 x 105 cells/well), and treated with various concentrations of pyroglutamyl dipeptides in the presence or absence of LPS for 24 h. Then, 20 μl of CellTiter 96® AQueous One Solution Reagent was added into each well. After 20 min, the absorbance at 490 nm was measured.

Measurement of nitric oxide release

The amount of nitrite in the cell-free culture supernatants was measured using a Griess reagent (Granger et al., 1996). Briefly, 100 μl of supernatant was mixed with an equivalent volume of Griess reagent [1:1 (v/v) of 0.1% N-1-naphthyl-ethylenediamine in distilled water and 1% sulfanilamide in 5% phosphoric acid] on a 96-well flat bottom plate. After 10 min, the absorbance at 550 nm was measured, and the amount of nitrite was calculated from the NaNO2 standard curve.

Measurement of TNF-α and IL-6 by ELISA

The concentrations of TNF-α and IL-6 in the culture supernatants and plasma in mice were determined by ELISA using Mouse TNF-α or IL-6 ELISA MAX Set Deluxe (Biolegend, CA, USA) in accordance with the manufacturer’s instructions.

Western blotting

Western blotting was performed as previously described (Hirai et al., 2007; Takahashi et al., 2002). In brief, RAW 264.7 cells were washed with PBS and placed immediately in a lysis buffer containing 20 μM Tris–HCl (pH 7.5), 15 mM NaCl, and a protease inhibitor cocktail set III (Calbiochem, CA, USA). The lysate was centrifuged at 15,000 rpm for 5 min, and the supernatant was stored for subsequent analysis. Protein concentration was determined using a Protein Assay Dye Reagent Concentrate (BioRad Laboratories, CA, USA) on the basis of the method of Bradford (1976). Fifteen micrograms of protein was separated by 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, MA, USA). After blocking, the membrane was incubated with anti-IgG (1/200) (sc-371: Santa Cruz Biotechnology, CA, USA), anti-JNK (1/1000) (#9258: Cell Signaling Technology, MA, USA), anti-pJNK (Thr183/Tyr185) (1/1000) (#9215: Cell Signaling Technology), anti-ERK (1/1000) (#4695: Cell Signaling Technology), anti-pERK (Thr202/Tyr204) (1/1000) (#9101: Cell Signaling Technology), anti-p-p38 (1/1000) (#9212: Cell Signaling Technology), or anti-p-p38 (Thr180/Tyr182) (1/200) (#9211: Cell Signaling Technology) antibody overnight, and then with a secondary antibody conjugated to horseradish peroxidase; anti-rabbit IgG (1/2000) (#W4011: Promega) for 1 h. The secondary antibody staining was visualized by chemiluminescence immunoassay using a chemiluminescent HRP substrate (Millipore).

Measurement for the binding of FITC-conjugated LPS to RAW 264.7 cells

RAW 264.7 cells were seeded on 96 well black plates (1 x 105 cells/ml) and pretreated with 400 or 800 μg/ml of pyroGlu-Leu for 4 h, and then incubated with 5 μg/ml of FITC-conjugated LPS (Sigma) and pyroGlu-Leu at the same doses as the pretreatment for 1 h. After washing the cells with PBS, the binding of FITC-LPS to the cell surface was measured on the basis of fluorescent intensity at 485/535 nm using Infinite F200, fluorescence plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Statistical analysis

The data are presented as mean ± SE of four replicants. Statistical comparison among groups were carried out using ANOVA and Williams’ or Shirley–Williams’ multiple comparison tests. Differences were considered significance at P < 0.05.

Results

Anti-inflammatory effect of pyroglutamyl dipeptides

First, we measured the cytotoxicity of each pyroglutamyl dipeptides. As shown in Fig. 2A, less than 90% of survival rate was observed in the cells treated with pyroGlu-Val, -Met, and -Phe even at 200 μg/ml. PyroGlu-Leu showed the lowest effect on the cell viability; no significant cytotoxic effect was observed in the cells treated with pyroGlu-Leu at the concentration below 400 μg/ml.

Next, we examined the effect of pyroglutamyl dipeptides on the LPS-induced inflammation in RAW 264.7 macrophages. PyroGlu-Leu, pyroGlu-Val, pyroGlu-Met, and pyroGlu-Phe were all suppressed LPS-induced secretion of NO (Fig. 2B), TNF-α (Fig. 2C), and IL-6 (Fig. 2D) from RAW 264.7 cells in a dose-dependent manner. Above all, pyroGlu-Leu significantly inhibited the secretion of all these inflammatory mediators even at the lowest dose (200 μg/ml) (Fig. 2B, C, D).

Suppression of IκBα degradation and MAPK phosphorylation by pyroGlu-Leu in LPS-stimulated RAW 264.7 cells

As pyroGlu-Leu most markedly inhibited the secretion of inflammatory mediators, we focused on the mechanism by which pyroGlu-Leu inhibits LPS-induced inflammation in macrophages. NF-κB and MAPK are known as the major signaling pathways that regulate the induction of TNF-α and IL-6 (Guha and Mackman, 2001). Therefore, the degradation of IκBα, which leads to the activation of NF-κB, and phosphorylation of MAPK: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38, were examined in RAW 264.7 cells treated with LPS. Although LPS treatment for 30 min markedly promoted IκBα degradation in RAW 264.7 macrophages, the degradation was inhibited by pyroGlu-Leu in a dose-dependent manner (Fig. 3). Furthermore, LPS-induced phosphorylation of JNK, ERK, and p38 were all
inhibited by pyroGlu-Leu treatment in a dose-dependent manner (Fig. 3).

**PyroGlu-Leu has no effect on the binding of LPS to RAW 264.7 macrophages**

Some kinds of peptides are reported to inhibit the cellular binding of LPS to macrophages (Huang et al., 2010; Nagaoka et al., 2001). Therefore, we examined the effect of pyroGlu-Leu on the binding of FITC-LPS to RAW 264.7 cells. Incubation of pyroGlu-Leu with FITC-LPS did not affect fluorescent intensity of cell surface (Fig. 4).
Discussion

Peptides play an important role in the biochemical and physiological functions of our body. Recently, various functional peptides are found in plants (Lico et al., 2012), animals (Redwan, 2009), or marine organisms (Aneiros and Garateix, 2004), and their health-promoting effects are expected. In the present study, focusing on pyroglutamyl peptides which had been found in the plasma of rats administered with WGH (Higaki-Sato et al., 2006), we evaluated their effects on inflammatory responses.

Inflammation is a host response to injury related to chemical or microbiological toxins. LPS is expressed in the outer membrane of gram-negative bacteria, and plays a key role in the initiation of inflammation by producing inflammatory mediators including NO, TNF-α, and IL-6 (Guha and Mackman, 2001). Our results indicated that all kinds of pyroglutamyl dipeptides we used in this study reduced the production of inflammatory signals to the nucleus (Guha and Mackman, 2001; Xiao et al., 2007). LPS stimulation induces the phosphorylation and activation of three types of MAPKs; JNK, ERK, and p38 MAPK (Guha and Mackman, 2001). JNK signaling regulates the expression of iNOS (Uto et al., 2005), whereas ERK signaling and p38 signaling upregulate iNOS expression and the production of proinflammatory cytokines including TNF-α and IL-6 in LPS-stimulated macrophages (Ajizian et al., 1999; Chan and Riches, 2001). On the other hand, Caivano (1998) reported that ERK and p38 are not rate-limiting for iNOS induction in RAW 264.7 macrophages. Our data showed that pyroGlu-Leu dose-dependently inhibited the degradation of h-Bx and phosphorylation of three types of MAPKs. These findings suggest that the anti-inflammatory properties of pyroGlu-Leu are probably due to the inhibition of LPS-stimulated activation of NF-κB via the suppression of h-Bx degradation and partly the phosphorylation of MAPKs in RAW 264.7 cells. On the other hand, pyroGlu-Leu did not interfere the binding of LPS to the cell surface of macrophages in the present study. Several anti-inflammatory peptides are reported to suppress LPS-induced activation of macrophages through the interaction with LPS, which results in the neutralization of LPS action (Nagaoka et al., 2001; Rosenfeld et al., 2008; Srivastava et al., 2012). On the other hand, it is also reported that relatively low molecular-weight peptides such as dipeptides or tripeptides can be imported into the cells by peptide transporters (Yang et al., 2002). Therefore, pyroGlu-Leu, a dipeptide, is considered to be transported into the cells and directly affect downstream signaling of TLR4 in macrophages.

Our research group recently reported that pyroGlu-Leu suppressed β-galactosamine (D-GalN)-induced hepatitis in rats (Sato et al., 2013). We have also found that pyroGlu-Leu decreases plasma TNF-α and IL-6 levels in LPS-administered mice (unpublished data). In D-GalN and LPS-administered animals, NF-κB and MAPKs are activated in macrophages in various tissues (He et al., 2011; Liu et al., 2013; Nakagawa and Maeda, 2012); therefore, pyroGlu-Leu is considered to exert anti-inflammatory activity in vivo by the same mechanism as in vitro. Recently, many kinds of functional peptides are demonstrated, but the problem is their instability or short-half life in vivo; many natural peptides are easily hydrolyzed and have limited availability. Further studies are required to clarify the availability and the effectiveness of pyroGlu-Leu in vivo.

Conclusion

Our study demonstrated that pyroGlu-Leu suppresses LPS-induced inflammation in RAW 264.7 macrophages. As pyroGlu-Leu did not affect the binding of LPS to the cell membrane, this dipeptide was considered to be incorporated into the cells and directly inhibit NF-κB and MAPK activation.

Conflict of interest statement

We have no conflict of interest other than that Shimmura Y is an employee of Nisshin Pharma Inc.

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