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Anti-Inflammatory Effect of Placenta Water-Soluble Extract on Macrophages

Miyuki Yokotani[1], Kosuke Nishi[1,2], Yoshiharu Sasaki[3], Takuya Sugahara[1,2,*]

[1] Graduate School of Agriculture, Ehime University, Matsuyama, Ehime 790-8566, Japan
[2] Food and Health Sciences Research Center, Ehime University, Matsuyama, Ehime 790-8566, Japan
[3] Fine Japan Co., Ltd., Higashiyodogawa-ku, Osaka 533-0021, Japan

* Corresponding author: mars95@agr.ehime-u.ac.jp; Tel.: +81-89-946-9863

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Abstract: The placenta is an organ attached to the lining of womb during pregnancy. It contains many kinds of substances such as amino acids, lipids, carbohydrates, vitamins, and minerals. It also has various health functions such as anti-allergy and anti-oxidant. We examined the anti-inflammatory effect of placenta water-soluble extract (PE) on lipopolysaccharide (LPS)-stimulated macrophages. PE significantly suppressed interleukin (IL)-6 and tumor necrosis factor (TNF)-α production by LPS-stimulated RAW264.7 cells and LPS-stimulated P-Mac without cytotoxicity. The gene expression levels of inflammatory cytokines in LPS-stimulated macrophages were significantly suppressed by PE. Then, the effect of PE on signal transduction for macrophage activation was evaluated. As a result, phosphorylation levels of ERK, JNK and p38 in MAPK cascade located at downstream of TLR4 signaling were decreased by PE treatment. In addition, heat-treatment of PE did not affect the inflammatory activity, so the active substance in PE is heat stable. As indicated here, our findings suggest that PE suppresses LPS-induced inflammation by inhibiting phosphorylation of MAPK cascade.

Keywords: RAW264.7, macrophage, anti-inflammatory, cytokine, MAPK

1. Introduction

The placenta is an organ attached to the lining of womb during pregnancy. It connects to fetus and provides oxygen and nutrients. In recent years, it is known that placenta has some health function such as anti-oxidant [1] and skin-whitening effects [2].

When infection occurs in the body, macrophages are induced to release inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-12, IL-8, and IL-6. These cytokines activate immune cells [3]. However, the excessive production of these inflammatory cytokines is to trigger many disease such as obesity, Alzheimer's disease, and cancer. It is also reported that many cancers arise from sites of infection, chronic irritation and inflammation. In this study, the anti-inflammatory effect of placenta water-soluble extract (PE) on macrophages was examined.

2. Materials and Methods

2.1. Preparation of placenta water-soluble extract

Placenta powder was provided by Fine Japan Co., Ltd (Osaka, Japan). Placenta powder was suspended in 10 mM sodium phosphate buffer (NaPB; pH 7.4) at 0.1 g/mL at 10°C for 24 h. After centrifugation at 15,000 × *g* at 4°C for 20 min, the supernatant was collected and adjusted to pH 7.4. The supernatant was then filtrated through a 0.22 μm membrane and used as PE. The protein concentration of PE was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

2.2. Cells and cell culture

A mouse macrophage-like cell line, RAW264.7 cells were purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). RAW264.7 cells were cultured in DMEM supplement with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS at 37°C under humidified 5% CO_2. RAW264.7 cells were detached using phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% ethylenediamine-*N, N, N′, N′*-tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) for the subsequent experiments.

2.3. Preparation of mouse primary peritoneal macrophages

Eight-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were injected with 3.0% of thioglycolate medium (2 mL/body) in the peritoneum. Four days after injection, the mice were sacrificed and injected with 3 mL of PBS in the peritoneum to collect thioglycolate-elicited peritoneal macrophages (P-Mac). The collected cells were centrifuged at 160 × *g* for 5 min at 4°C, and the cell pellet was washed with PBS and centrifuged again. The cell pellet was suspended in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS. Cells were seeded into a 24-well culture plate (BD Falcon, Franklin Lakes, NJ, USA) at 1.0×10^6 cells/well and cultured at 37°C under humidified 5% CO_2. After cultivation for 1 h, the cells were washed with PBS three times to remove unattached cells such as neutrophils. P-Mac were cultured at 37°C under humidified 5% CO_2 overnight and used for the subsequent experiments.

2.4. Determination of cytokine levels in culture medium

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 96-well culture plate (Corning, Corning, NY, USA) at 6.0×10^4 cells/well and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, the cells were treated with 200 μL of 10% FBS-DMEM containing 100 ng/mL of lipopolysaccharides (LPS; Sigma-Aldrich, St. Louis, MO, USA) and various concentrations of PE or 10 mM NaPB as a control and incubated for 6 h. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. P-Mac suspended in 10% FBS-RPMI 1640 medium were seeded into a 24-well culture plate at 1.0×10^6 cells/well and cultured at 37°C under humidified 5% CO_2. After washing with PBS, the cells were treated with 1.0 mL of 10% FBS-RPMI 1640 medium containing 100 ng/mL of LPS and various concentrations of PE or 10 mM of NaPB as control and incubated for 24 h. Blank cells were treated with 10% FBS-RPMI 1640 medium containing 10 mM NaPB alone. After the incubation, the concentrations of IL-6 and TNF-α in the culture media were measured by enzyme-linked immunosorbent assay (ELISA) using mouse IL-6 ELISA kit (BioLegend, San Diego, CA, USA) and mouse TNF-α ELISA kit (eBioscience, San Diego, CA, USA), respectively, according to the manufacturer's instructions.

2.5. Cell viability

Cytotoxicity of PE extract was examined using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) according to manufacturer's instructions after collecting the culture media from each well for ELISA.

2.6. Real time RT-PCR

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 48-well culture plate (BD Falcon) at 1.5×10^5 cells/well and cultured at 37°C overnight under humidified 5% CO_2. After washing with PBS, the cells were treated with 500 μL of 10% FBS-DMEM containing 100 ng/mL of LPS and various concentrations of PE or 10 mM NaPB as control and incubated for 6 h. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. P-Mac suspended in 10% FBS-RPMI 1640 medium was seeded into a 24-well culture plate at 1.0×10^6 cells/well and cultured at 37°C under humidified 5% CO_2. The cells were treated with 1.0 mL of 10% FBS-RPMI 1640 medium containing 100 ng/mL of LPS and various concentrations of PE or 10 mM NaPB as control and incubated for 24 h. Total

RNA was then isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the manufacturer's instructions and used as a template for cDNA synthesis with MMLV-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-$(dT)_{20}$ primer (Toyobo, Osaka, Japan). A real-time PCR mixture, with a final volume of 20 μL, consisted of Thunderbird SYBR qPCR Mix (Toyobo), 10 pmol of a forward primer, 10 pmol of a reverse primer, and 0.1 μg of a cDNA sample. Thermal cycling conditions were 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. PCR products were measured on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated based on the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Expression of the β-actin gene was used as an endogenous control. Specific oligonucleotide sequences for each gene are as follows. Mouse β-actin: sense, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and antisense, 5'-ATGGAGCCACCGATCCACA-3'; mouse TNF-α: sense, 5'-CTACTCCCAGGTTCTCTTCAA-3' and antisense, 5'-GCAGAGAGGAGGTTGACTTTC-3'; mouse IL-6: sense, 5'-AAGCCAGAGTCCTTCAGAGAGAT-3' and antisense, 5'-TTGGATGGTCTTGGTCCTTAGC-3'; mouse inducible nitric oxide synthase (iNOS): sense, 5'-CCAAGCCCTCACCTACTTCC-3' and antisense, 5'-CTCTGAGGGCTGACACAAGG-3'.

2.7. Nitric oxide production assay

RAW264.7 cells suspended in 10% FBS-DMEM were inoculated into a 96-well cell culture plate at 1.0×10^4 cells/well and cultured for 12 h. The cells were treated with 200 μL of 10% FBS-DMEM containing 100 ng/mL of LPS and various concentrations of PE or 10 mM NaPB as control and incubated for 24 h. The concentration of nitric oxide (NO) was measured by Griess Reagent System (Promega) according to the manufacturer's instructions.

2.8. Immunoblot analysis

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 35 mm dish (BD Falcon) at 1.0×10^6 cells/dish and cultured at 37°C for overnight under humidified 5% CO_2. After washing with PBS, the cells were treated with 2.0 mL of 10% FBS-DMEM containing 100 ng/mL of LPS and 6200 μg/mL of PE or 10 mM NaPB as control and incubated for 15 min. Blank cells were treated with 10% FBS-DMEM containing 10 mM NaPB alone. Cytosolic and nuclear proteins were prepared using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. Denatured proteins were then separated using SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described [4].

2.9. Heat-treatment of PE

To examine the effect of heat-treated PE on cytokine production by LPS-stimulated RAW264.7 cells, PE was heated at 100°C for 20 min and used for assay of activity.

2.10. Statistical analysis

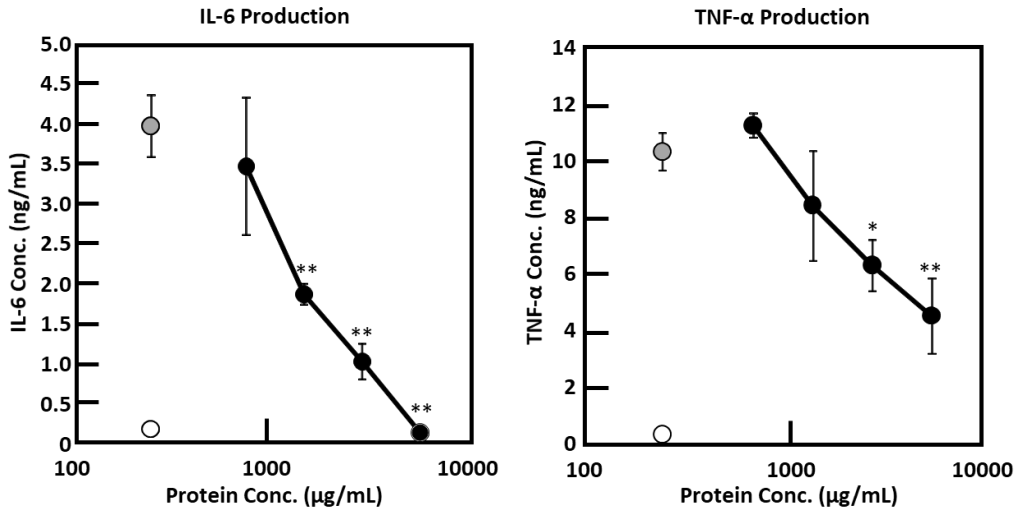
Data obtained were expressed as mean ± standard deviation. One way ANOVA followed by Tukey-Kramer test was used to assess the statistical significance of the difference. Values with $^{*}p < 0.05$, $^{**}p < 0.01$, were considered statistically significant.

3. Results

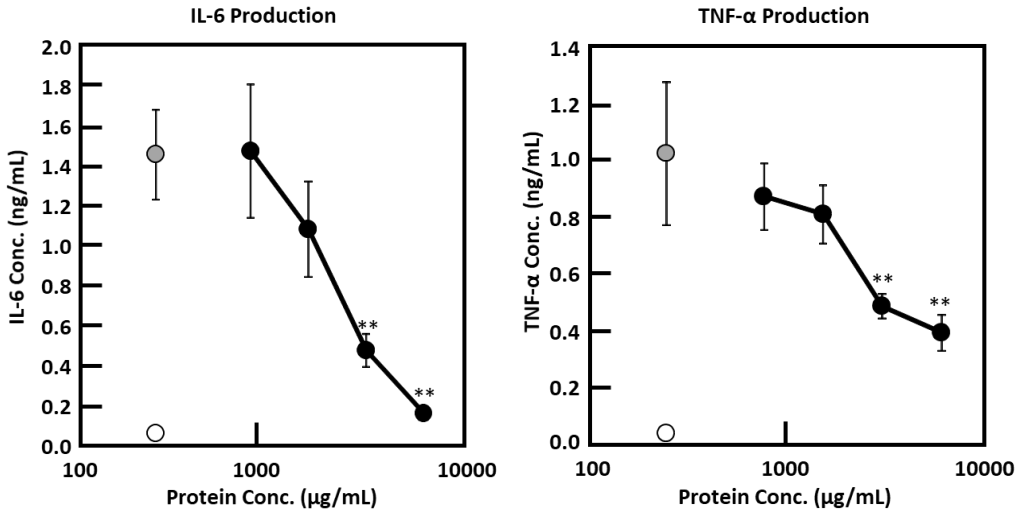
3.1. Effect of PE on cytokine production by RAW264.7 cells and P-Mac

The effect of PE on cytokine production by RAW264.7 cells and P-Mac was first examined. As shown in Figure 1, PE significantly inhibited cytokine production by both RAW264.7 cells and P-Mac in dose-dependent manners. In addition, it showed that PE has no significant cytotoxicity to either cell

culture within the tested concentrations (data not shown). From these results, further experiments were performed at 6200 μg protein/mL or lower concentrations of PE.



(a) RAW264.7 cells

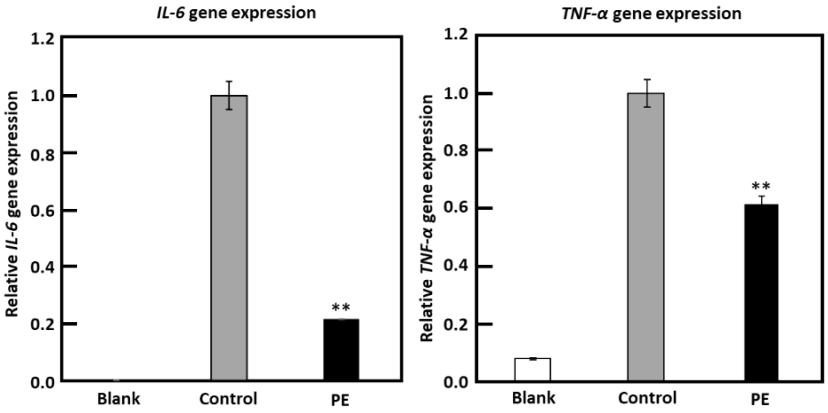


(b) P-Mac

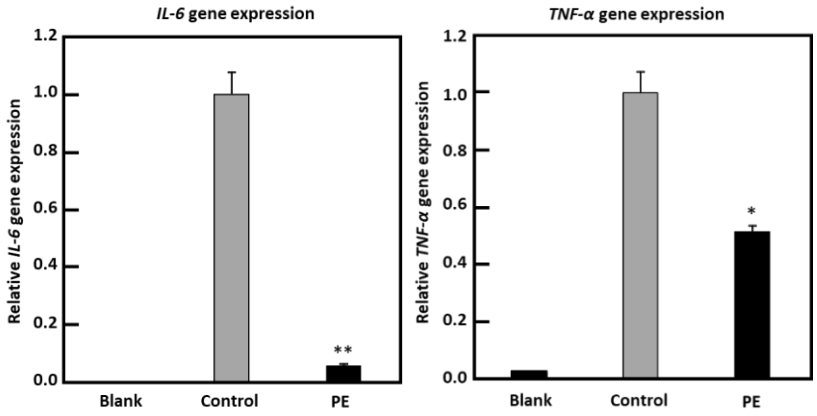
Figure 1. Effect of PE on cytokine production by RAW264.7 cells and P-Mac. (**a**) RAW264.7 cells and (**b**) P-Mac were incubated with 100 ng/mL of LPS and various concentrations of PE (closed circle) or 10 mM NaPB as control (gray circle). Blank cells (open circle) were treated with 10 mM NaPB alone. After incubation, the concentrations of IL-6 and TNF-α in the culture media were measured by ELISA. Experiments were performed in triplicate, and error bars indicate as the mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, against control by Tukey-Kramer test.

3.2. Effect of PE on cytokine gene expression levels in RAW264.7 cells and P-Mac

As described above, PE inhibited the production of IL-6 and TNF-α by both RAW264.7 cells and P-Mac. Hence, the effect of PE on mRNA expression levels of these cytokines was examined. As shown in Figure 2, PE significantly inhibited the mRNA expression levels of IL-6 and TNF-α in both RAW264.7 cells and P-Mac. It is suggested that the effect of PE on cytokine production is due to the downregulation of cytokine gene expression.



(a) RAW264.7 cells



(b) P-Mac

Figure 2. Effect of PE on cytokine gene expression levels by RAW264.7 cells and P-Mac. (**a**) RAW264.7 cells and (**b**) P-Mac were incubated with 100 ng/mL of LPS and various concentrations of PE (closed circle) or 10 mM NaPB as control (gray circle). Blank cells (open circle) were treated with 10 mM NaPB alone. After incubation, the gene expression levels of IL-6 and TNF-α were measured by real time RT-PCR. Experiments were performed in duplicate, and error bars indicate as the mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, against control by Tukey-Kramer test.

3.3. Effect of PE on NO production and mRNA expression of iNOS in RAW264.7 cells

NO is recognized as one of the most versatile compounds in the immune system and produced by macrophages activated by cytokines and microbial compounds. Therefore, we examined the effect of PE on NO production by LPS-stimulated RAW264.7 cells. The result showed that PE significantly inhibited NO production by LPS-stimulated RAW264.7 cells (data not shown). In addition, PE also suppressed the mRNA expression of iNOS in the cells, suggesting that PE inhibits NO production by suppressing iNOS gene expression.

3.4. Effect of PE on signaling pathways involved in macrophage activation

To examine the involvement of MAPK and NF-κB signaling pathways in the inhibition of the IL-6 and TNF-α production by treating with PE, the protein levels of signal molecules were evaluated by immunoblot analysis. As a result, the phosphorylation levels of ERK, JNK and p38 were decreased by PE treatment, whereas PE did not affect the translocation of NF-κB into the nucleus in LPS-stimulated RAW264.7 cells (data not shown). These results suggested that PE inhibits inflammatory cytokine production through downregulation of MAPK cascade.

3.5. Effect of heat-treated PE on cytokine production by RAW264.7 cells

To investigate whether the active substance in PE is heat-stable or not, PE was heated at 100°C for 20 min. The result showed that the cytokine production-suppressive activity of PE was not affected by heat treatment (data not shown), suggesting that the active substance in PE is heat-stable.

4. Discussion

In this study, we firstly measured cytokine concentration in the culture media to assess the anti-inflammatory activity of PE. PE significantly inhibited the production of IL-6 and TNF-α by both RAW264.7 cells and P-Mac in dose-dependent manners. PE also significantly suppressed the gene expression levels of IL-6 and TNF-α in both RAW264.7 cells and P-Mac. These results suggested that PE inhibits the cytokine production by suppressing the transcription process. In addition, PE inhibited NO production by suppressing the mRNA expression of iNOS in LPS-stimulated RAW264.7 cells, suggesting that PE inhibits NO production by suppressing iNOS gene expression.

There are two signaling pathways involved in the production of cytokines and NO by macrophages, namely, MAPK and NF-κB cascades. Therefore, we examined the effect of PE on MAPK and NF-κB cascades. The result showed that the phosphorylation levels of ERK, JNK, and p38 in MAPK cascade located at downstream of TLR4 signaling were decreased by PE treatment. On the other hand, PE did not affect the translocation of NF-κB into the nucleus in the LPS-stimulated cells. These results indicated that the activity of PE is due to the downregulation of MAPK cascade.

Furthermore, we investigated the properties of active substance in PE. The result showed that heat-treated PE has the inhibitory effect on cytokine production by LPS-stimulated RAW264.7 cells, suggesting that the active substance in PE is heat-stable.

5. Conclusions

Although macrophages have an important role to release cytokines when inflammation occurs, the excess of producing these cytokines causes diseases. In this study, we found that PE has anti-inflammatory effect on macrophages. In addition, our findings suggested that PE suppresses LPS-induced inflammation by inhibiting the activation of MAPK cascade. Taken together, these data indicate that placenta would be a beneficial functional food with anti-inflammatory effect on macrophages.

References

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