The effects of erucin on inflammatory mediators and antioxidant enzymes expression in TNF-α-stimulated human oral epithelial cells

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Abstract

Periodontitis is a chronic inflammatory disease induced by periodontal disease-causing bacteria. It has been shown that excessive immune response against bacteria is involved in periodontal tissue destruction including alveolar bone resorption. Erucin is a biologically active substance found in cruciferous plants such as arugula, and is classified as an isothiocyanate. No previous studies have attempted to use erucin in the treatment of periodontitis, and there are no papers that have examined the effects of erucin on periodontal resident cells. The purpose of this study was to analyze the effects of erucin on the production of inflammatory and antioxidant mediators produced by tumor necrosis factor (TNF)-α-stimulated TR146 cells, an oral epithelial cell line, including its effects on signaling molecules. Our results indicate that erucin suppresses interleukin (IL)-6 and CXC-chemokine ligand (CXCL)10 production and vascular cell adhesion molecule (VCAM)-1 expression in TNF-α-stimulated TR146 cells. In addition, erucin induced the production of the antioxidant enzymes, Heme Oxygenase (HO)-1 and NAD(P)H quinone dehydrogenase (NQO)1 in TR146 cells. Furthermore, erucin suppressed TNF-α-stimulated nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT)3, and p70S6K-S6 signaling pathways in TR146 cells. We have shown that erucin has anti-inflammatory effects on oral epithelial cells and also induces the production of antioxidant mediators. These results suggest that erucin may provide a new anti-inflammatory agent that can be used in the treatment of periodontitis.

Introduction

Periodontitis is an inflammatory disease caused by periodontopathogenic bacteria, and excessive production of inflammatory mediators at the site of periodontitis lesions has been shown to induce periodontal tissue destruction (1, 2). It has been reported that inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α produced by leukocytes such as macrophages infiltrating into the periodontitis lesions stimulate gingival epithelial cells and gingival fibroblasts to produce additional inflammatory mediators (2).

IL-6 is mainly produced by periodontal resident cells and has been suggested to cause alveolar bone resorption by participating in osteoclast differentiation (3). CXC chemokine ligand (CXCL)10 is also produced by periodontal resident cells and has been implicated in periodontal tissue destruction by causing infiltration and accumulation of Th1 cells in periodontal lesions (4), and adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 have been reported to be expressed on periodontal resident cells, and have been implicated in the localization of leukocytes in periodontitis lesions, and stimulation of leukocytes at the site of periodontitis lesions, leading to further exacerbation of inflammation (5).

Oxidative stress caused by reactive oxygen species and free radicals, which cause degeneration of proteins and nucleic acids, has been implicated in lifestyle diseases such as diabetes (6), atherosclerosis (7), and hypertension (8). It has also been reported that oxidative stress is involved in the pathogenic mechanism of periodontal disease, which is also a lifestyle disease. Heme Oxygenase (HO)-1 and NAD(P)H quinone dehydrogenase (NQO)1 are antioxidant mediators produced by human cells, and it has been suggested that finding bioactive substances that promote their production may lead to the treatment of lifestyle diseases including periodontal disease (10,

11).

Erusin is a bioactive substance found in cruciferous plants such as arugula and is classified as an isothiocyanate. Various bioactive activities of erucin have been reported, including anticancer and anti-inflammatory effects. As an anticancer effect, erucin has been reported to inhibit the growth of breast cancer cells (12). As for anti-inflammatory effects, it has been reported that erucin suppresses TNF-α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 expression in macrophages induced by lipopolysaccharide (LPS) stimulation (13). However, there are no reports examining the effects of erucin on periodontal tissue component cells, and there are no attempts to use erucin in the treatment of periodontitis.

The aim of this study was to elucidate the effects of erucin on the expression of inflammatory mediators and antioxidant enzymes induced in oral epithelial cells by TNF-α stimulation, which is a proinflammatory cytokine. We also sought to determine the effects of erucin on signaling pathways activated by TNF-α stimulation in human oral epithelial cells.

Materials and Methods

Cell Culture

Human oral epithelial cells (TR146 cells) were kindly provided by Dr Mark Herzberg (Minnesota University). TR146 cells were grown in Ham's F12 media (Nakarai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 1 mmol/L sodium pyruvate (Gibco, Grand Island, MI, USA), and antibiotics (penicillin G, 100 units/mL; streptomycin, 100 μg/mL; Gibco) at 37 °C in a humid environment with 5% CO_2. The cells were taken for subculture when they were subconfluent using a 0.25% trypsin- ethylenediaminetetraacetic acid

(EDTA) solution.

Cytotoxic Assay

Cell Count Reagent SF (Nakarai Tesque) was used to test cell viability. TR146 cells were seeded in 96-well plates, and cultured for 2 days. The media was withdrawn after 2 days, and 90 μL of Ham's F12 medium containing various doses of erucin (Cayman Chemical, Ann Arbor, MI, USA) was added. The cells were then cultured for an additional 24 hours. We added 10 μL of Cell Count Reagent SF, allowed the cells to remain for 2 hours, and used a microplate reader to detect the absorbance at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

TNF-α (100 ng/mL: Peprotech, Rocky Hill, NJ, USA) with or without erucin (1.5625, 3.125, 6.25, 12.5, 25 or 50 μM) was applied to TR146 cells for 24 hours. The concentrations of IL-6 and CXCL10 in TR146 cell culture supernatant were measured using DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) as directed by the manufacturer.

Western blot analysis

TR146 cells were cultured in 12-well plates, and total protein was collected in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) after TNF-α (100 ng/mL) stimulation for 15, 30, or 60 minutes with or without erucin (25 μM) pretreatment for 1 hour, or TNF-α (100 ng/mL) stimulation for 24 hours with or without erucin (12.5, 25, or 50 μM). The protein concentrations in the lysates were determined using the BCA Protein Assay Kit (TaKaRa, Shiga, Japan). A similar quantity of protein was loaded onto a 4-20% SDS-polyacrylamide gel electrophoresis (PAGE) gel and electrotransferred to a polyvinylidene difloride membrane. The membranes were blocked for 1 hour at room temperature with 1% skim milk before being incubated with primary antibodies against

VCAM-1 (1/1000 dilution, Biolegend, San Diego, CA, USA), HO-1 (1/1000 dilution, Cell Signaling Technology), NQO1 (1/1000 dilution, Cell Signaling Technology), Phospho-NF-κB p65 (1/1000 dilution, Cell Signaling Technology), NF-κB p65 (1/1000 dilution, Cell Signaling Technology), Phospho-IκB-α (1/1000 dilution, Cell Signaling Technology), IκB-α (1/1000 dilution, Cell Signaling Technology), Phospho-STAT3 (1/2000 dilution, Cell Signaling Technology), STAT3 (1/2000 dilution, Cell Signaling Technology), Phospho-p70S6K (1/1000 dilution, Cell Signaling Technology), p70S6K (1/1000 dilution, Cell Signaling Technology), Phospho-S6 (1/2000 dilution, Cell Signaling Technology), S6 (1/4000 dilution, Cell Signaling Technology), or Glyceraldehyde-3-phosphate dehydrogenase (1/8000 dilution, Cell Signaling Technology) at 4 °C overnight. The membranes were then washed and treated with a secondary antibody conjugated with horseradish peroxidase (HRP) (1/10000 dilution, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 hour. Protein bands on western blot images were detected using the ECL Prime Western-blotting detection system (Cytiva, Tokyo, Japan). Image J software was used to calculate the density of western blot analysis bands (version 1.52p: NIH, Bethesda, MD, USA).

Statistical Analysis

In order to determine statistical significance, we performed one-way analysis of variance (ANOVA) followed by a post hoc Tukey-Kramer test, and p values less than 0.05 were considered significant.

Results

The analysis of cytotoxic effects of erucin on TR146 cells

At the first of this study, we examined at what concentrations of erucin is cytotoxic to TR146 cells. The results showed that concentrations of erucin lower than 50 μM were not cytotoxic to TR146

cells (Fig.1). In this study, we decided to use concentrations of erucin below 50 μM.

The effect of erucin on IL-6 and CXCL10 production in TNF-α-stimulated TR146 cells

We have reported that TR146 cells produces IL-6 and CXCL10 upon TNF-α stimulation (14, 15). Therefore, we investigated whether erucin affects TNF-α-induced IL-6 and CXCL10 production. We demonstrated that IL-6 and CXCL10 production in TNF-α-stimulated TR146 cells was decreased in a concentration-dependent manner by erucin (Fig.2).

The effect of erucin on VCAM-1 expression in TNF-α-stimulated TR146 cells

In our previous report, we showed that TNF-α induces VCAM-1 expression in TR146 cells (15). Therefore, we decided to examine whether erucin influences the VCAM-1 expression induced by TNF-α in TR146 cells. We showed that erucin reduced TNF-α-induced VCAM-1 expression in TR146 (Fig.3).

The effect of erucin on NF-κB activation in TNF-α-stimulated TR146 cells

Previous studies have shown that NF-κB is involved in the production of inflammatory mediators (16). In this study, we investigated whether erucin affects phosphorylation of NF-κB p65 and IκB-α and degradation of IκB-α in TNF-α-stimulated-TR146 cells. The results showed that 25 μM erucin inhibited the phosphorylation of NF-κB p65 and IκB-α induced by TNF-α stimulation (Fig.4). The degradation of IκB-α in TR146 cells stimulated with TNF-α for 15 min or 30 min was inhibited by 25 μM erucin. (Fig.4).

The effect of erucin on STAT3 phosphorylation in TNF-α-stimulated TR146 cells

We have previously reported that TNF-α stimulation induces phosphorylation of STAT3 in TR146 cells (15). Therefore, we examined whether erucin might affect STAT3 phosphorylation. We found

that 25 μM erucin inhibited TNF-α-induced STAT3 phosphorylation (Fig.5).

The effect of erucin on p70S6K and S6 phosphorylation in TNF-α-stimulated TR146 cells

We previously demonstrated that the p70S6K-S6 pathway is activated by TNF-α stimulation and that 6-Methylsulfinylhexyl isothiocyanate (6-MSITC), an isothiocyanate found in horseradish, inhibits the activation of p70S6K-S6 pathway (14). We hypothesized that erucin might have a similar effect. In this study, we found that 25 μM erucin treatment attenuated TNF-α-induced phosphorylation of p70S6K and S6 in TR146 cells (Fig.6).

The effect of erucin on HO-1 and NQO1 expression in TNF-α-stimulated TR146 cells

It has been reported that oxidative stress is involved in the pathogenesis of periodontitis (9). Therefore, we hypothesized that the increase of antioxidant mediators, HO-1 and NQO1, in the localized periodontitis lesions is important to suppress the progression of periodontitis. We found that erucin treatment increased HO-1 and NQO1 expression in TR146 cells (Fig.7).

Discussion

Periodontal disease is an inflammatory disease caused by periodontopathogenic bacteria, and local administration of antibiotics is used to treat periodontitis. However, the use of large doses of antibiotics is feared to lead to an increase in the number of resistant bacteria (17), making the search for new anti-inflammatory substances an urgent issue. In this study, erucin was found to have an anti-inflammatory effect on periodontal tissue component cells. This finding suggests that erucin may have a potential to be used for the treatment of periodontitis.

This study demonstrated that erucin can inhibit the production of inflammatory mediators such as IL-6, CXCL10, and VCAM-1 in human oral epithelial cells. Several reports have investigated the

anti-inflammatory effects of erucin. It was reported that TNF-α, VCAM-1, COX-2, and E-cadherin expression in human vascular endothelial cells stimulated with LPS was suppressed by pretreatment with erucin (18). Another report demonstrated that erucin decreases LPS-induced iNOS, COX-2, TNF-α, IL-6 and IL-1β production in RAW 264.7 cells (13). Our present findings and previous reports have shown that erucin has anti-inflammatory effects on a variety of cells, including epithelial cells, vascular endothelial cells, and macrophages. Since not only oral epithelial cells but also vascular endothelial cells and inflammatory cells are present at the site of periodontitis lesions, the anti-inflammatory effect on various cells may be advantageous in the treatment of periodontitis. In this study, we demonstrated that erucin suppresses several signaling transduction pathways. Previous papers have also examined the effects of erucin on signaling transduction pathways. Cho et al. reported that erucin inhibited IκB-α degradation in LPS-stimulated macrophages similar to our findings (13). Although there are no reports of erucin inhibiting STAT3 or p70S6K-S6 activation, we have already reported that other isothiocyanates, 6-MSITC and iberin, inhibit STAT3, p70S6K, and S6 phosphorylation (14, 15). These results suggest that inhibition of NF-κB, STAT3, and the p70S6K-S6 signaling pathway may be a common effect of isothiocyanates. Further studies are needed to prove this hypothesis.

We found that erucin increases HO-1 and NQO1 expression and suppresses the NF-kB pathway. Antioxidant enzymes such as HO-1 and NQO1 have been reported to influence the activation of the NF-κB signaling pathway. Bellezza et al. reported that HO-1 activation inhibited NF-κB nuclear translocation in prostate cancer cells (19). Ahn et al also showed that NF-κB activation, IκB-α phosphorylation and degradation, induced by TNF-α and LPS was abolished in NQO1 deleted cells (20). Our study also suggests that increased HO-1 and NQO1 may influence NF-κB activation.

We found that 25 μM erucin had an anti-inflammatory effect on TR146 cells and induced the expression of antioxidant enzymes. Furthermore, 25 μM erucin inhibited the activation of various signaling pathways. However, concentrations of erucin lower than 25 μM exhibited anti-inflammatory effects in this experiment. The effect of even lower concentrations on TR146 cells may also need to be investigated.

Conclusions

In conclusion, this report shows that erucin has an anti-inflammatory effect on human oral epithelial cells and also induces the expression of antioxidant enzymes. Therefore, it is necessary to examine whether or not erucin has the same effect on other periodontal tissue constituent cells. In addition, in vivo studies using animal models are also considered necessary.

Disclosure statement

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, Y.H.; investigation, M.S., Y.H., I.H.; methodology, M.S., Y.H., I.H., and K.O.; writing—original draft, Y.H.; writing—review and editing, K.O. and K.H. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data in this manuscript are available from the corresponding author on reasonable request.

Figure Legends

Fig.1. The effect of erucin on cell viability of TR146 cells.

TR146 cells were seeded on 96-well cell culture plates, grown for two days, and then treated for 24 hours with erucin (1.5625-50 μM). The vitality of cells was determined using Cell Count Reagent SF. The data are presented as the mean SD of four independent experiments. * = $P<0.05$, significantly different from TR146 cells not treated with erucin.

Fig.2. The effect of erucin on TNF-α-induced production of CXCL10 and IL-6.

TR146 cells were grown for 24 hours with TNF-α (100 ng/ml) with or without erucin (1.5625-50 μM). CXCL10 and IL-6 levels in the supernatant were measured using the ELISA kits indicated in the Materials and Methods section. The data are presented as the mean SD of three independent experiments. * = $P<0.05$, significantly different from TNF-α-stimulated TR146 cells without erucin.

Fig. 3. The effect of erucin on VCAM-1 expression in TNF-α-stimulated TR146 cells

TR146 cells were pretreated for one hour with erucin (12.5, 25, or 50 μM) before being stimulated with TNF-α (100 ng/ml). The lysates were taken 24 hours after stimulation. The expression of VCAM-1 was investigated using Western blot analysis. (A) Representative Western blot image of the expression of VCAM-1 and GAPDH. (B) Quantification of protein expression by densitometry analysis of Western blots. Data are expressed as the mean ± SD of 3 independent experiments. (*= $P<0.05$ vs TNF-α-stimulated TR146 cells without erucin)

Fig.4. The effect of erucin treatment on the activation the NF-κB pathway in TNF-α stimulated TR146 cells.

TR146 cells were pretreated for one hour with erucin (25 μM) before being stimulated with TNF-α for 15, 30, or 60 minutes, and the phosphorylation of NF-κB p65 and IκB-α and the degradation of IκB-α were measured using Western immunoblotting. (A) Representative Western blot image of the expression of phospho-NF-κB p65, total NF-κB p65, phospho-IκB-α, IκB-α, and GAPDH. (B, C, and D) Quantification of protein expression by densitometry analysis of Western blots. Data are expressed as the mean ± SD of 3 independent experiments. (*= P<0.05)

Fig.5. The effect of erucin treatment on the activation of the STAT3 pathway in TNF-α stimulated TR146 cells.

TR146 cells were pretreated for one hour with erucin (25 μM) before being stimulated with TNF-α for 15, 30, or 60 minutes, and the phosphorylation of STAT3 was evaluated using Western immunoblotting. (A) Representative Western blot image of the expression of phospho-STAT3, total STAT3, and GAPDH. (B) Quantification of protein expression by densitometry analysis of Western blots. Data are expressed as the mean ± SD of 3 independent experiments. (*= P<0.05)

Fig.6. The effect of erucin treatment on the activation of a p70S6K-S6 pathway in TNF-α stimulated TR146 cells.

TR146 cells were pretreated for one hour with erucin (25 μM) before being stimulated with TNF-α for 15, 30, or 60 minutes, and the phosphorylation of p70S6K and S6 was measured using Western immunoblotting. (A) Representative Western blot image of the expression of phospho-p70S6K, total p70S6K, phospho-S6, total S6, and GAPDH. (B, C) Quantification of protein expression by

densitometry analysis of Western blots. Data are expressed as the mean ± SD of 3 independent experiments. (*= $P<0.05$)

Fig. 7. Effects of erucin on HO-1 and NQO1 expression in TNF-α-stimulated TR146 cells

TR146 cells were pretreated for one hour with erucin (12.5, 25, or 50 μM) before being stimulated with TNF-α (100 ng/ml). The lysates were taken 24 hours after stimulation. The expression of HO-1 and NQO1 was investigated using Western blot analysis. (A) Representative Western blot image of the expression of HO-1, NQO1, and GAPDH. (B, C) Quantification of protein expression by densitometry analysis of Western blots. Data are expressed as the mean ± SD of 3 independent experiments. (*= $P<0.05$ vs TNF-α-stimulated TR146 cells without erucin)

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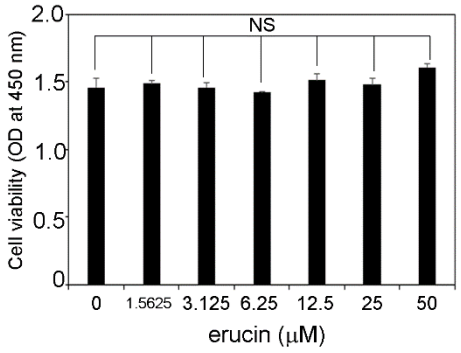
NS
2.0
1.5
1.0
0.5
0
Cell viability (OD at 450 nm)
0
1.5625
3.125
6.25
12.5
25
50
erucin (μM)

Fig. 1

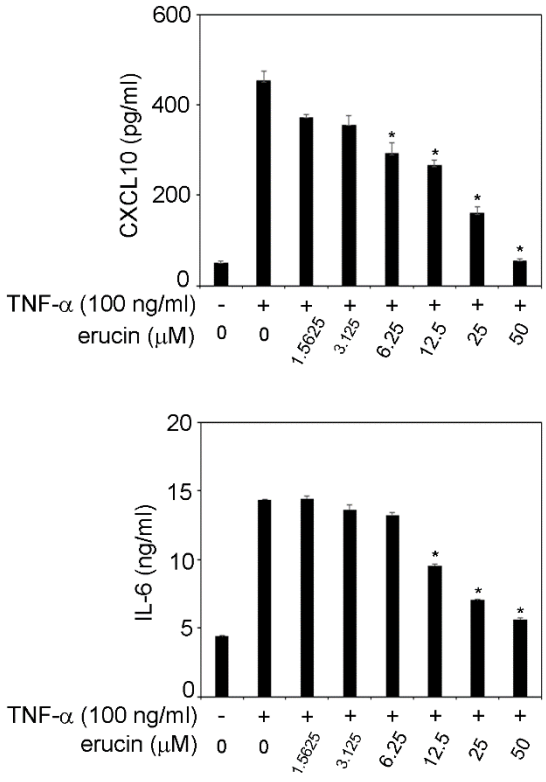
600
400
200
0
CXCL10 (pg/ml)
TNF-α (100 ng/ml) - + + + + + + +
erucin (μM) 0 0 1,5625 3,125 6,25 12,5 25 50
20
15
10
5
0
IL-6 (ng/ml)
TNF-α (100 ng/ml) - + + + + + + +
erucin (μM) 0 0 1,5625 3,125 6,25 12,5 25 50

Fig. 2

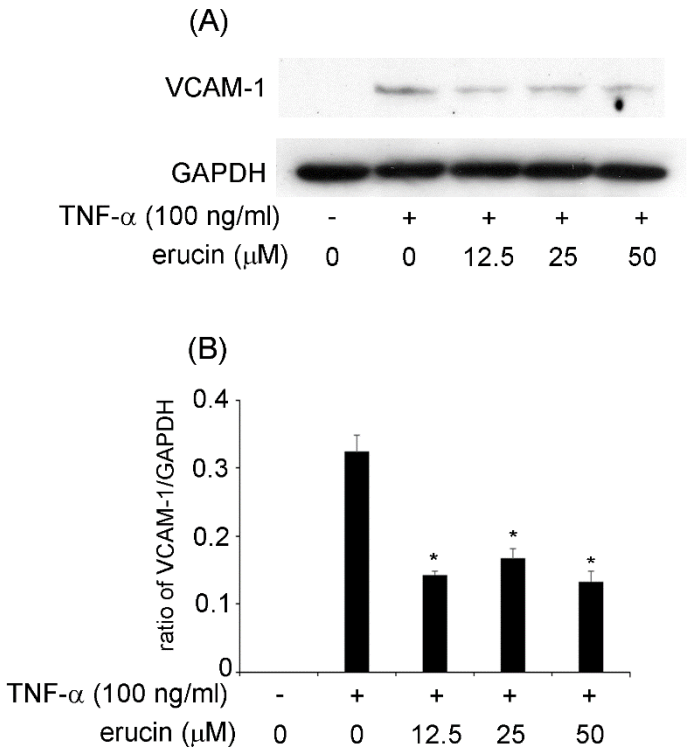
(A)
VCAM-1
GAPDH
TNF-α (100 ng/ml) - + + + +
erucin (μM) 0 0 12.5 25 50
(B)
ratio of VCAM-1/GAPDH
0.4
0.3
0.2
0.1
0
*
*
*
TNF-α (100 ng/ml) - + + + +
erucin (μM) 0 0 12.5 25 50

Fig. 3

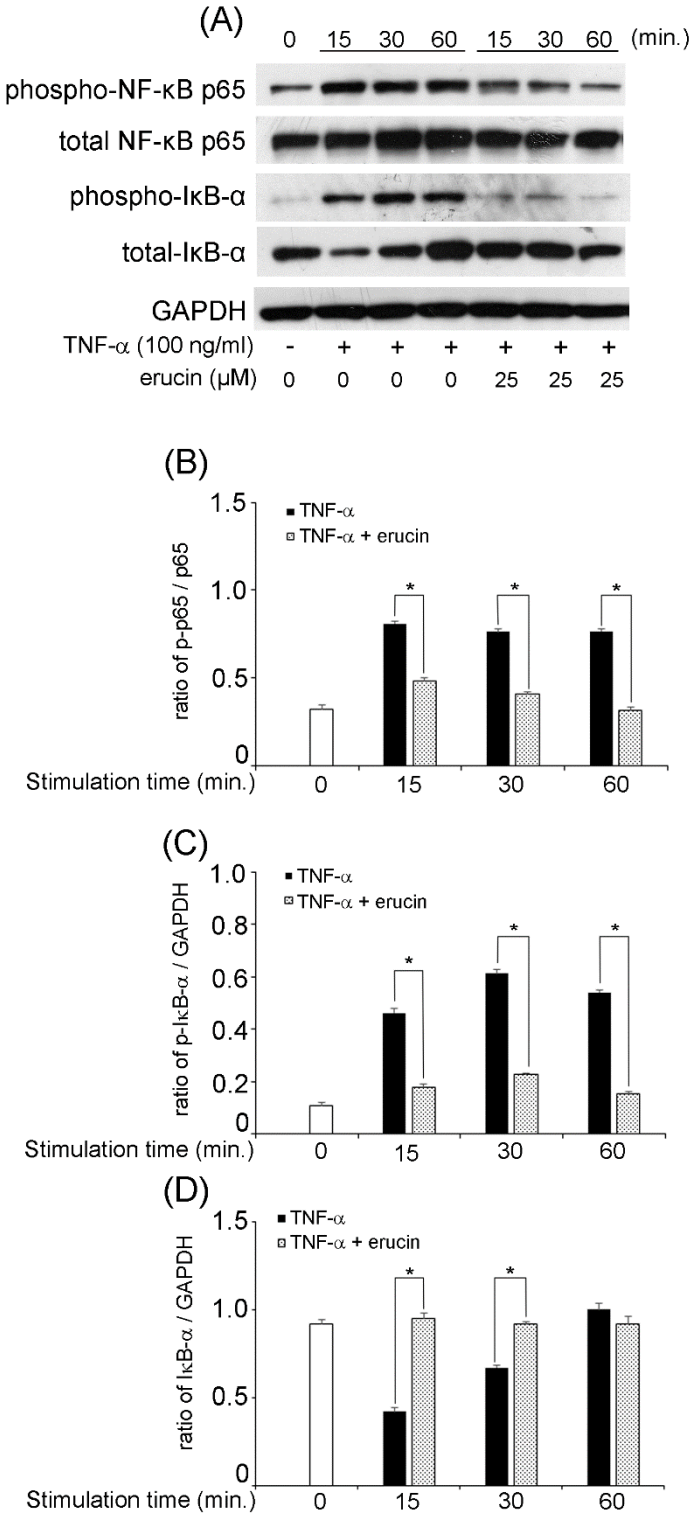
(A)
0 15 30 60 15 30 60 (min.)
phospho-NF-κB p65
total NF-κB p65
phospho-IκB-α
total-IκB-α
GAPDH
TNF-α (100 ng/ml) - + + + + + +
erucin (μM) 0 0 0 0 25 25 25
(B)
ratio of p-p65 / p65
TNF-α
TNF-α + erucin
Stimulation time (min.) 0 15 30 60
(C)
ratio of p-IκB-α / GAPDH
TNF-α
TNF-α + erucin
Stimulation time (min.) 0 15 30 60
(D)
ratio of IκB-α / GAPDH
TNF-α
TNF-α + erucin
Stimulation time (min.) 0 15 30 60

Fig. 4

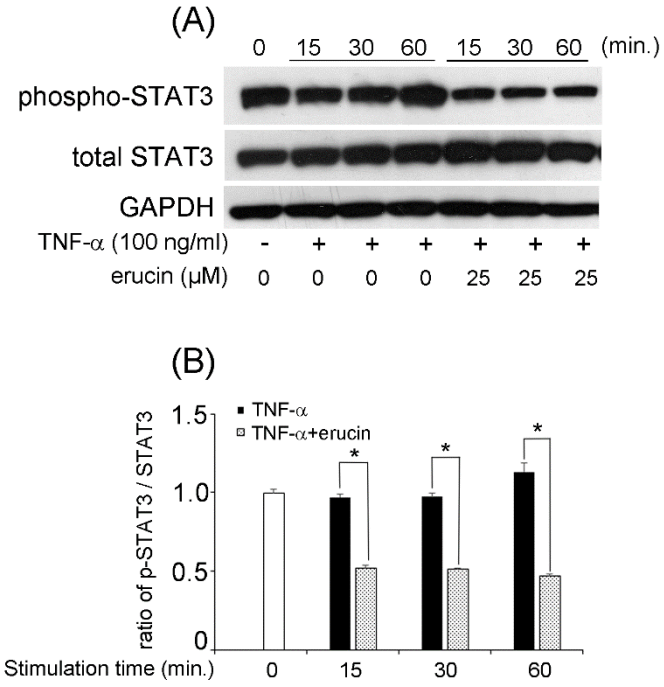
(A)
0 15 30 60 15 30 60 (min.)
phospho-STAT3
total STAT3
GAPDH
TNF-α (100 ng/ml) - + + + + + +
erucin (μM) 0 0 0 0 25 25 25
(B)
TNF-α
TNF-α+erucin
ratio of p-STAT3 / STAT3
1.5
1.0
0.5
0
*
*
*
Stimulation time (min.) 0 15 30 60

Fig. 5

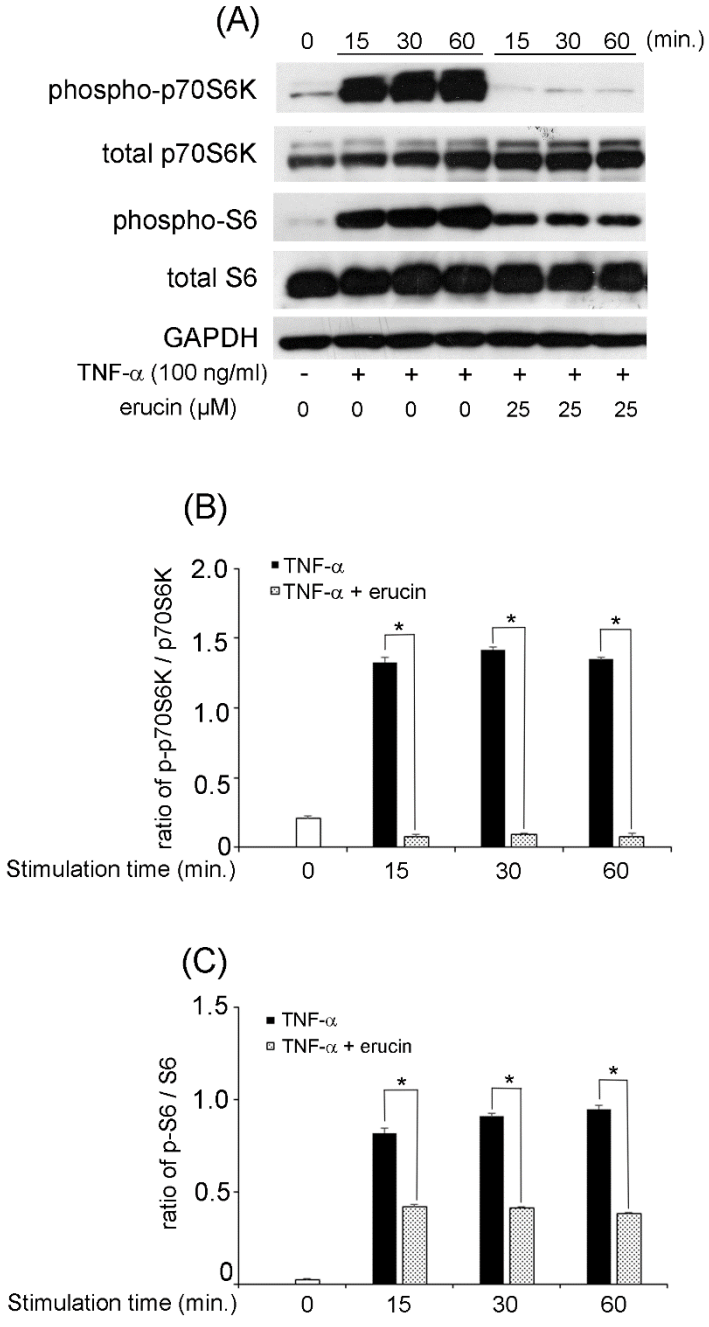
(A)
0 15 30 60 15 30 60 (min.)
phospho-p70S6K
total p70S6K
phospho-S6
total S6
GAPDH
TNF-α (100 ng/ml) - + + + + + +
erucin (μM) 0 0 0 0 25 25 25
(B)
TNF-α
TNF-α + erucin
ratio of p-p70S6K / p70S6K
2.0
1.5
1.0
0.5
0
Stimulation time (min.) 0 15 30 60
(C)
TNF-α
TNF-α + erucin
ratio of p-S6 / S6
1.5
1.0
0.5
0
Stimulation time (min.) 0 15 30 60

Fig. 6

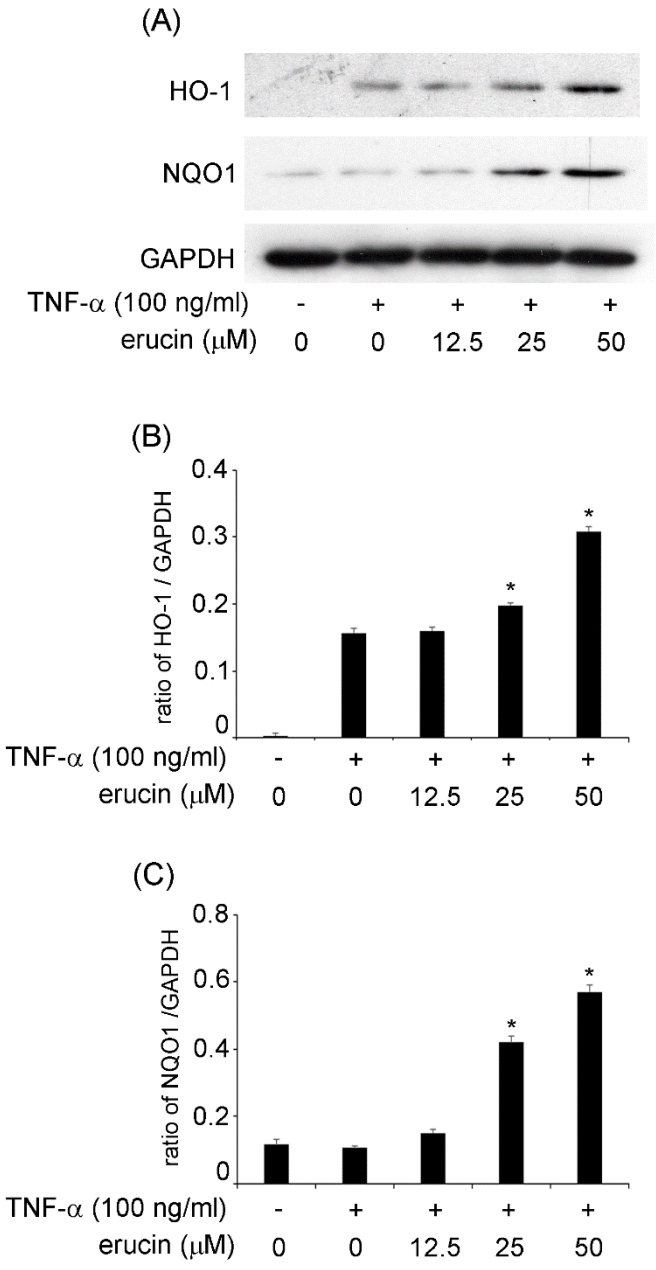
(A)
HO-1
NQO1
GAPDH
TNF-α (100 ng/ml) - + + + +
erucin (μM) 0 0 12.5 25 50
(B)
ratio of HO-1 / GAPDH
0.4
0.3
0.2
0.1
0
*
*
TNF-α (100 ng/ml) - + + + +
erucin (μM) 0 0 12.5 25 50
(C)
ratio of NQO1 /GAPDH
0.8
0.6
0.4
0.2
0
*
*
TNF-α (100 ng/ml) - + + + +
erucin (μM) 0 0 12.5 25 50

Fig. 7