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Isoflavone genistein and daidzein up-regulate LPS-induced inducible nitric oxide synthase activity through estrogen receptor pathway in RAW264.7 cells

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Dai, daidzein
E2, 17β-estradiol
ELISA, enzyme-linked immunosorbent assay
ER, estrogen receptor
FBS, fetal bovine serum
Gen, genistein
iNOS, inducible nitric oxide synthase
L-NAME, N^G-nitro-L-arginine methyl ester
LPS, lipopolysaccharide
PBS, phosphate buffered saline
PI3K, phosphatidylinositol 3-kinase
TNF-α, tumor necrosis factor α

ABSTRACT

Isoflavones, such as genistein and daidzein, are found in abundance in soybeans. These plant-derived substances have estrogenic activities and can bind to the estrogen receptors (ERs). In this study, we investigated that the effects of 17β-estradiol (E2), genistein and daidzein on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) activity in RAW264.7 cells. We found that these isoflavones significantly increased lipopolysaccharide-induced NO production and iNOS expression as much as E2 at physiological concentrations. Moreover, E2 and isoflavone enhanced the production of tumor necrosis factor-α that is one of the important cytokines regarding NO production. The enhancing effects of E2 and isoflavones on NO production were markedly inhibited by not only N^G-nitro-L-arginine methyl ester (an inhibitor of NOS), but also ICI 182780 (ERs antagonist). Two types of ERs were identified as ERα and ERβ. An ERα agonist could increase iNOS expression in RAW264.7 cells, while an ERβ agonist could not. In conclusion, our results suggest E2, genistein and daidzein activate iNOS, and then up-regulate NO production. This enhancing effect is aroused through ERα pathway in RAW264.7 cells.

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1. Introduction

Macrophage-derived nitric oxide (NO) is important for host defense and microbial killing [1,2]. NO is generated from L-arginine and molecular oxygen by the activation of NO synthase (NOS) [3]. Inducible NOS (iNOS) expression can be induced by lipopolysaccharide (LPS) and/or cytokines in a variety of cells, including macrophage, bone-marrow microglial cells and smooth muscle cells [4–6]. In addition, it has been reported that iNOS expression is regulated at multiple levels, including transcription, post-transcription, and translation [7,8].

Soybeans are particularly rich in isoflavones, such as genistein and daidzein. Because of the structural similarity to natural estrogens, they have estrogenic activities, for example, prevention of bone loss, decrease the level of low-density lipoprotein and affect thymic weight [9–11]. Moreover, the low incidence of osteoporosis in Asian women has been attributed to diet rich in isoflavones [12,13]. 17β-Estradiol (E2) has a lot of important roles in immune system [14]. We have showed that E2 enhances IgM production by mouse splenocytes [15], and up-regulates LPS-induced interferon-γ (IFN-γ) production by activating natural killer cells [16].

Several cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are important for regulation of the NO production [1]. E2 mediates IL-1β production and promoter activity [17,18]. However, it is poorly understood that the effects of E2 and isoflavones on NO production in macrophages. We were taken a variety of estrogenic compounds such as genistein and daidzein from soy foods [19,20]. Therefore, it is necessary to investigate effects of E2 and isoflavones on the immune system. In this study, we revealed the effects of E2 and isoflavones on LPS-induced NO and TNF-α production of RAW264.7 cells. In addition, the effects on iNOS activity and expression levels were investigated.

Principal functions of E2 are expressed through estrogen receptors (ERs). It is well known that there are two ER subtypes, ERα and ERβ [21,22]. Genistein and daidzein have affinity for ERα and ERβ [23,24]. We have shown that E2 mediates IgM production through ERα and ERβ pathway [15]. In this study, we also investigated the relation between estrogens and ERs in activation of iNOS.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium was derived from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Phenol red-free RPMI 1640 medium, E2, lipopolysaccharide (LPS), anti-β-actin polyclonal antibody and horseradish peroxidase (HRP)-conjugated anti-rabbit antibody were derived from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Biofluids (Rockville, MD). Genistein and daidzein were purchased from Fujicco (Kobe, Japan). 4,4′,4″-(4-propyl-[1*H*]-prazole-1,3,5-triyl)*tris*phenol (PPT), 2,3-*bis*-(4-hydroxyphenyl)-propionitrile (DPN) and ICI 182780 were purchased from TOCRIS (Bistrol, UK). Block Ace was purchased from Dainihon Pharmaceutical (Osaka, Japan). An anti-iNOS polyclonal antibody was purchased from Merck (Tokyo, Japan). An anti-Akt antibody and HRP-conjugated anti-goat antibody were purchased from Santa Crzu Biotechnology, Inc. (Santa Crzu, CA). An anti-phospho-Akt was obtained from Cell signaling Technology, Inc. (Beverly, MA). An anti-mouse TNF-α antibody was obtained from Endogen (Woburn, MA). Biotinylated anti-mouse TNF-α antibody was obtained from Biosource (Camarillo, CA). HRP-conjugated anti-mouse antibody and HRP conjugated streptavidin were purchased from Zymed (San Francisco, CA). Napthalethylenediamine dihydrochloride, sulfanilamine and Blocking one were obtained from Nacalai tesque (Kyoto, Japan). Trizol was purchased from Invitrogen (Carlsbad, CA). ECL Western detection regents, oligo-dT20 primer, dNTP and M-MLV reverse transcriptase were purchased from Amersham Biosciences (Piscataway, NJ). RNase inhibitor was purchased from Takara Bio (Shiga, Japan).

2.2. Cell and cell culture

RAW264.7 cells were inoculated at 1×10^5 cells/ml in RPMI 1640 medium containing 5% FBS for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. And then, the medium was changed to phenol red-free RPMI 1640 containing 5% charcoal-treated FBS with E2, genistein, daidzein, ICI 182780, PPT or DPN in presence of LPS 20 ng/ml of LPS. For the preparation of whole cell lysates, cells were washed with phosphate buffered saline (PBS), and lysed in a buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5 mM Sodium orthovanadate, 50 mM NaF, and 5 mM EDTA-2Na. After centrifugation at 15,000 × *g* for 15 min at 4 °C, the supernatant was collected, and the protein concentration was determined.

2.3. Western blot analysis

Equal amount of protein (5 μg/lane) were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany). The membrane was washed with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl), containing 0.1% Tween20 (TBST). The membrane was blocked with Blocking one solution at 37 °C for 1 h. Following blocking, the membrane was incubated with antibodies against iNOS, Akt, phospho-Akt (1:2000) or β-actin (1:10,000), and then continuously incubated with appropriate HRP-conjugated antibodies. The bands were developed by using of the ECL Western detection reagents.

2.4. Measurement of NO

After treatment of E2, genistein or daidzein in the presence of LPS, RAW264.7 cell culture medium was saved for the measurement of nitrite, an indicator of NO production. Briefly [25], culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1% napthalethylenediamine dihydrochloride, 1% sulfanilamine and 2.5% H_3PO_4). Absorbance of the mixture was then measured at 535 nm. The concentration of

nitrite was converted into sodium nitrite concentration as a standard.

2.5. Enzyme-linked immunosorbent assay

The amount of TNF-α in culture medium was measured by sandwich enzyme-linked immunosorbent assay (ELISA). An anti-mouse TNF-α antibody was used to fix TNF-α. Antibody solution diluted at 500 times by PBS was added to a 96-well plate, and incubated for 2 h at 37 °C. After washing with 0.05% Tween20-PBS (T-PBS) three times, each well was blocked with 25% Block Ace solution for 2 h at 37 °C. Following blocking reaction, each well was treated with 50 μl of culture supernatant for 1 h at 37 °C. The well was then treated with biotinylated anti-mouse TNF-α antibody diluted at 1000 times by 10% Block Ace for 1 h at 37 °C. HRP-conjugated streptavidin solution diluted at 1000 times by 10% Block Ace solution was added to each well at 100 μl. Then, 0.6 mg/ml of 2,2′-azino-bis(ethylbenzothazoline-6-sulfonic acid diammonium salt) (ABTS) dissolved in 0.03% H_2O_2–0.05 M citrate buffer (pH 4.0) was added to the well at 100 μl, and the absorbance at 405 nm was measured after the addition of 1.5% oxalic acid to terminate the coloring reaction at 100 μl. The well was rinsed three times with T-PBS between each step.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 cells were harvested by centrifugation and washed twice in PBS. Total cellular RNA was isolated using the Trizol reagent according to the manufacturer's instructions. Ten micrograms of total RNA was denatured at 70 °C with 1 μl oligo-dT20 primer (0.5 mg/ml) in 13.8 μl final volume. Primers-RNA mixes were cooled on ice for 10 min, 2 μl dNTPs (10 mM), 0.1 μl M-MLV reverse transcriptase (20 units), 0.1 μl RNase inhibitor (10 units) and 4 μl 5 × buffer were added in 20 μl final volume. The resultant cDNA samples were subjected to 25 cycles of PCR amplification in the presence of specific sense and antisense primers. Mouse β-actin cDNA was amplified as an internal control. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min (β-actin, iNOS and TNF-α), and DNA synthesis at 72 °C for 1 min. Sequences for the PCR primers sizes and expected amplification product sizes are as follows: for the β-actin: sense 5′-TGGAATCCTGTGGCATC-CATGAAAC-3′, and antisense 5′-TAAAACGCAGCTCAGTAA-CAGTCCG-3′; for the iNOS: sense 5′-GACAAGCTGCATGTGA-CATC-3′, and antisense 5′-GCTGGTAGGTTCCTGTTGTT-3′; for the TNF-α: sense 5′-TCCCCAAAGGGATGAGAAGTTC-3′, and antisense 5′-TCATACCAGGGTTTGAGCTCAG-3′. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel.

2.7. Statistical analysis

All data were expressed as the mean ± S.D. Statistical significance was analyzed by Student's *t*-test (Figs. 1A, 2, 3 and 4) or Scheffe's *F*-test (Fig. 6). Each value of $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ was considered to be statistically significant.

3. Results

3.1. Effects of 17β-estradiol on NO production and iNOS expression in RAW264.7 cells

To examine the effect of E2 on NO production in RAW264.7 cells, the cells were incubated with E2 at physiological concentrations (10^{-11} to 10^{-7} M) (Fig. 1A). E2 significantly increased NO production in the medium at the concentrations as low as 10^{-10} M. The production of NO was increased in a dose-dependent manner by E2 stimulation. The maximal effect was observed at a concentration of 10^{-8} M. This observation clearly suggests that physiological concentration of E2 significantly induces the up-regulation of NO production.

To elucidate the underlying mechanisms of the regulation of NO synthesis in the macrophages by E2, we demonstrated whether E2 mediates the protein expression level of iNOS or not. As the result of that, the iNOS expression was markedly enhanced in an E2 dose-dependent fashion (Fig. 1B). The maximal iNOS expression was achieved at 10^{-8} M E2. However, the NO production and iNOS expression were not detected in the absence of LPS stimulation (data not shown).

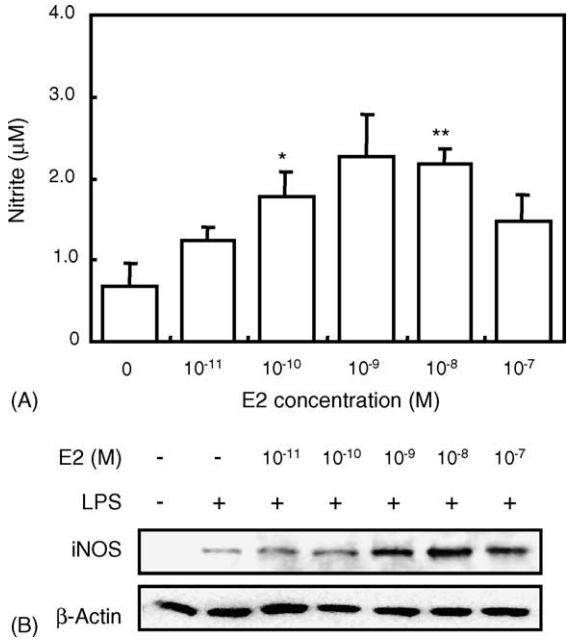

Fig. 1 – Effect of 17β-estradiol on LPS-induced NO production and iNOS expression in RAW264.7 cells. (A) RAW264.7 cells were treated with E2 (10^{-11} to 10^{-7} M) in the presence of 20 ng/ml LPS for 24 h. NO production was determined by measuring the accumulation of nitrite in the medium. Data are mean ± S.D. ($n = 3$). Data with asterisk marks are significantly different from the values in the control group at $^{*}p < 0.05$ or $^{**}p < 0.01$. (B) RAW264.7 cells were treated with E2 (10^{-11} to 10^{-7} M) in the presence of 20 ng/ml LPS for 24 h. Whole cell lysates were prepared, and analyzed for iNOS or β-actin by Western blotting.

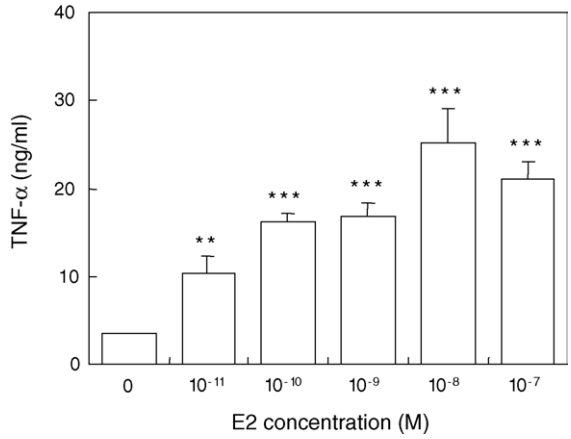

Fig. 2 – Effect of 17β-estradiol on TNF-α production in RAW264.7 cells. RAW264.7 cells were treated with E2 (10^{-11} to 10^{-7} M) in the presence of 20 ng/ml LPS for 24 h. The level of TNF-α in culture medium was measured by ELISA. Data are mean ± S.D. ($n = 3$). Data with asterisk marks are significantly different from the values in the control group at $^{**}p < 0.01$ or $^{***}p < 0.001$.

3.2. *Effect of 17β-estradiol on TNF-α production in RAW264.7 cells*

TNF-α is one of the important cytokines and mediates the stimulation of NO production. Then, it was examined the effect of E2 on TNF-α production under the LPS stimulation. E2 significantly increased TNF-α production dose-dependently (Fig. 2). The maximal effect occurred at a concentration of 10^{-8} M, at which NO production was maximally stimulated.

3.3. *Effects of isoflavones on NO and TNF-α production in RAW264.7 cells*

To examine whether soy bean isoflavones, genistein and daidzein, mediate NO production in RAW264.7 cells or not, the cells were incubated with isoflavones at the concentrations of 10^{-8} to 10^{-6} M. Genistein enhanced NO production at concentrations of 10^{-7} and 10^{-6} M, while daidzein stimulated from 10^{-8} to 10^{-6} M (Fig. 3).

The effects of genistein and daidzein on TNF-α production in RAW264.7 cells were determined. As the result, genistein and daidzein obviously increased TNF-α production. The maximal effects of genistein and daidzein were achieved at 10^{-7} and 10^{-6} M, respectively (Fig. 4).

3.4. *Effect of isoflavone on iNOS activity in RAW264.7 cells*

To determine whether the NO production stimulating effects of E2, genistein and daidzein are related to iNOS activity or not, the iNOS expression level in RAW264.7 cells was investigated under the stimulation of these compounds. The cells were treated with E2 (10^{-8} M), genistein (10^{-6} M) or daidzein (10^{-6} M). These isoflavones enhanced LPS-induced iNOS expression as much as E2 in RAW264.7 cells (Fig. 5A). However, in the absence of LPS stimulation, the iNOS expression was not detected by isoflavone stimulation (data not shown).

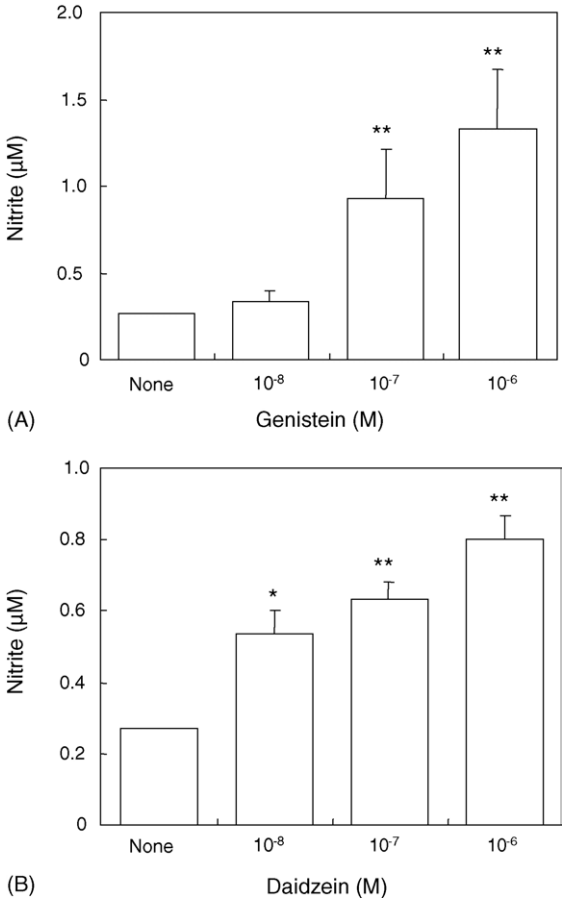

Fig. 3 – Effect of isoflavone genistein and daidzein on LPS-induced NO production in RAW264.7 cells. RAW264.7 cells were treated with genistein or daidzein (10^{-8} to 10^{-6} M) in the presence of 20 ng/ml LPS for 24 h. NO production was determined by measuring the accumulation of nitrite in the medium. Data are mean ± S.D. ($n = 3$). Data with asterisk marks are significantly different from the values in the control group at $^{*}p < 0.05$ or $^{**}p < 0.01$.

Moreover, the effects of E2 and isoflavones on iNOS and TNF-α mRNA expression in RAW264.7 cells was determined. None of these compounds stimulated iNOS mRNA and TNF-α mRNA expressions, even though the production levels of these proteins were increased (Fig. 5B).

L-NAME is an inhibitor of iNOS activity. The effect of L-NAME on NO production stimulated by E2, genistein or daidzein was evaluated. RAW264.7 cells were incubated with E2 or isoflavones in the presence of L-NAME. As the result, the effects of E2 and isoflavones on NO production were inhibited by L-NAME (Fig. 6).

3.5. *17β-Estradiol and isoflavones mediate iNOS expression through estrogen receptors*

It is important to investigate that the effects of E2, genistein, and daidzein on the regulation of iNOS expression are mediated by ERs. RAW264.7 cells were pretreated with ICI 182780, ER antagonist, for 30 min, and then each compound

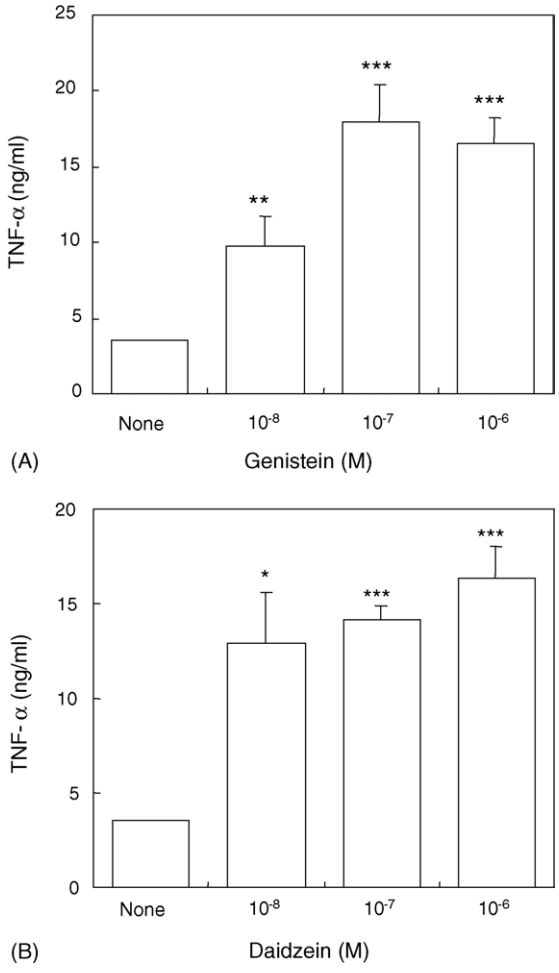

Fig. 4 – Effect of isoflavone genistein and daidzein on TNF-α production in RAW264.7 cells. RAW264.7 cells were treated with genistein and daidzein (10^{-8} to 10^{-6} M) in the presence of 20 ng/ml LPS for 24 h. The level of TNF-α in culture medium was measured by ELISA. Data are mean ± S.D. ($n = 3$). Data with asterisk marks are significantly different from the values in the control group at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

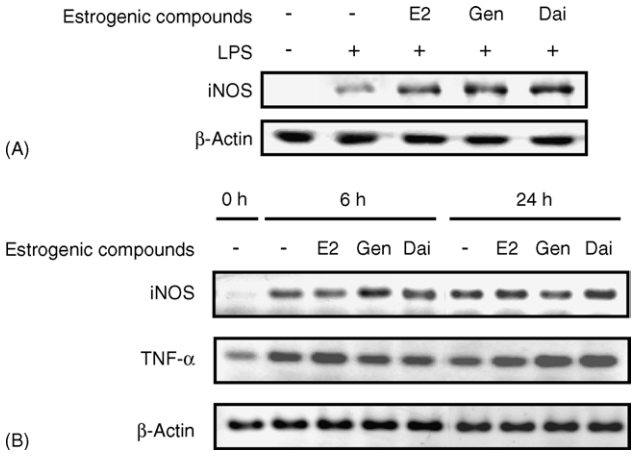

Fig. 5 – Effect of 17β-estradiol and isoflavone on iNOS and TNF-α mRNA expression in RAW264.7 cells. (A) RAW264.7 cells were treated with E2 (10^{-8} M), genistein or daidzein (10^{-6} M) in the presence of 20 ng/ml LPS for 24 h. Whole cell lysates analyzed for iNOS or β-actin by Western blotting. (B) RAW264.7 cells were treated with E2 (10^{-8} M), genistein or daidzein (10^{-6} M) in the presence of 20 ng/ml LPS for 0, 6 and 24 h. Total RNA were prepared, and analyzed for iNOS, TNF-α or β-actin by RT-PCR.

was added to the medium. The effects of these compounds on iNOS expression were inhibited by ICI 182780 (Fig. 7A).

Two subtypes of ER were identified, i.e. ERα and ERβ [21,22]. To investigate which ER subtype mediates the effects of isoflavones on LPS-induced iNOS expression, RAW264.7 cells were stimulated by ERα or ERβ agonists. The cells were incubated with ERα agonist, PPT (10^{-8} to 10^{-6} M) or ERβ agonist, DPN (10^{-8} to 10^{-6} M) under the LPS stimulation. PPT enhanced LPS-induced iNOS expression at 10^{-7} and 10^{-6} M as much as E2. However, DPN did not affect LPS-induced iNOS expression level (Fig. 7B).

4. Discussion

In previous study, we have demonstrated isoflavones have an estrogen like activity and mediate cytokine production by splenocytes isolated from C57BL/6N mice [26]. Estrogens can modulate NO production from eNOS in aortic rings [27], and endothelial cells [28,29]. E2 increases iNOS mRNA expression in macrophage [30]. However, the role of isoflavones in the regulation of NO synthesis in macrophage has been poorly revealed. It is important to understand the role of isoflavones in inflammatory reaction because isoflavones could act like estrogens, and we take it from foods. TNF-α is one of the most

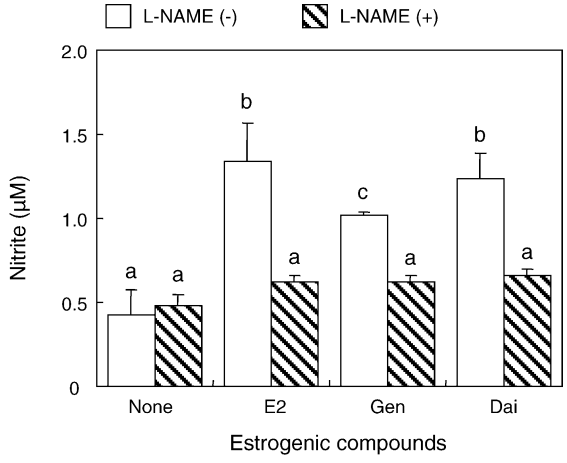

Fig. 6 – Effect of 17β-estradiol and isoflavone on NO production in presence of NOS inhibitor. RAW264.7 cells were treated with E2 (10^{-8} M), genistein or daidzein (10^{-6} M) in the presence of L-NAME (500 μM) and 20 ng/ml LPS for 24 h. NO production was determined by measuring the accumulation of nitrite in the medium. Data are mean ± S.D. ($n = 3$). Letters that are different from each other denote significant difference at $^{*}p < 0.05$.

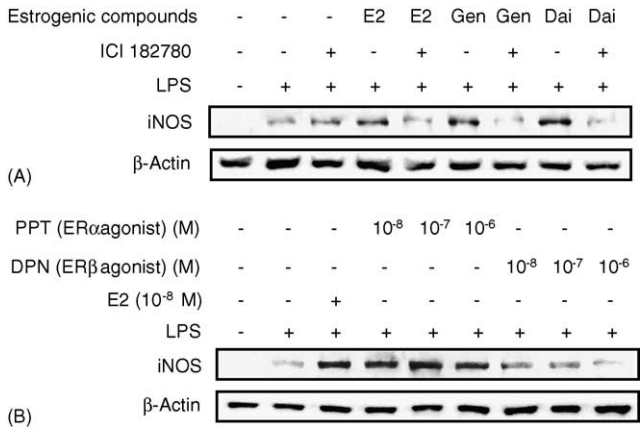

Fig. 7 – 17β-Estradiol and isoflavone mediate LPS-induced iNOS expression through estrogen receptors in RAW264.7 cells. (A) RAW264.7 cells were incubated with E2 (10^{-8} M), genistein (10^{-6} M) or daidzein (10^{-6} M) in presence of 20 ng/ml LPS for 24 h. The cells were pretreated with ICI 182780 (10^{-6} M) for 30 min before E2, genistein or daidzein application. Whole cell lysates were prepared, and analyzed for iNOS or β-actin by Western blotting. (B) RAW264.7 cells were incubated with E2 (10^{-8} M), PPT or DPN (10^{-8} to 10^{-6} M) in presence of 20 ng/ml LPS for 24 h. Whole cell lysates were prepared, and analyzed for iNOS or β-actin by Western blotting.

important regulatory cytokines and mediates a variety of cell functions, including stimulation of NO production in oxidative stress or chronic inflammation [1,31]. In this study, it was investigated that the effect of E2 and isoflavones on NO and TNF-α production in RAW264.7 cells under the LPS stimulation.

E2 and isoflavones enhanced LPS-induced NO and TNF-α production. The maximal effect of E2 was observed at the concentration of 10^{-8} M, which is equivalent to the circulating concentration of postmenopausal women receiving hormone replacement therapy [32]. The effects of genistein and daidzein were maximized at 10^{-7} and 10^{-6} M, respectively. The concentration range is close to the circulating concentration of women taken soy-fed human infants [11]. These results suggest that both of E2 and isoflavones affect RAW264.7 cells at physiological concentration. Daidzein enhanced NO and TNF-α productions at lower concentration than genistein. Recently, it was reported that the order of efficacy was estradiol > daidzein > genistein in osteoclastic differentiation [33]. In addition, genistein is an inhibitor of TNF-α, whereas daidzein induced TNF-α production in presence of LPS/IFN-γ stimulation in RAW264.7 cells [8]. Contrary to previous report, our results indicate that both genistein and daidzein increased TNF-α production of RAW264.7 cells under the LPS stimulation. However, concentration ranges of isoflavones tested here was quite different from those reported previously (16–500 μM).

NO is synthesized from L-arginine by iNOS in macrophages [34,35]. E2 and isoflavones increased LPS-induced iNOS expression of RAW264.7 cells. The molecular regulation of iNOS expression is primarily regulated at the transcription process [7,8]. In this study, however, the level of iNOS mRNA expression was not affected by E2 and isoflavones. The effects of E2 and isoflavones on NO production were inhibited by iNOS inhibitor, L-NAME. These results suggest that E2, genistein and daidzein enhance NO production by activating iNOS.

According to the classical hypothesis, the cellular effects of estrogens have been considered as a transcription factors mediated by intracellular. The structures of isoflavones, such as genistein and daidzein, are similar to that of E2, therefore they can bind weakly to both ERα and ERβ [36,37]. In this study, contribution of ERs for regulation of iNOS activity was examined by using an ER antagonist ICI 182780 which blocks forming ER dimmer and decomposes ERs [38]. ICI 182780 inhibited the effects of E2 and isoflavones. This suggests that up-regulation of LPS-induced NO production by E2 and isoflavones is mediated through ERs pathway.

It was reported that the expression of ERβ mRNA is increased by E2 exposure more than ERα mRNA expression in rat peritoneal macrophages [29]. We previously reported that E2 enhanced IgM production through both of ERα and ERβ mediation in mouse splenocytes [15]. In this study, it was cleared that only ERα agonist up-regulates iNOS expression in RAW264.7 cells. This means that ERα is more important than ERβ for iNOS expression, Wang et al. reported that there is an important ERβ-mediated response for osteoclastic differentiation in RAW264.7 cells [33]. Our findings are consistent with that report.

Recent reports are strongly suggesting that iNOS expression is linked to activation of intracellular signaling proteins, PI3K/Akt pathway [4,39,40]. Then the effect of E2 on the activation of Akt, a well-known target of PI3K, was examined. However, E2 has no effect on activation of Akt activity (data not shown). It is now under investigation that which signaling pathways is association with the iNOS activation by E2 and isoflavones.

In conclusion, E2, genistein and daidzein up-regulated LPS-induced NO production at the physiological concentrations. This effect of E2 and isoflavones was derived from activation of iNOS. Moreover, these compounds accelerated LPS-induced iNOS expression through ERα.

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