**Note**

17β-Estradiol Increased the Expression of Daintain/AIF-1 in RAW264.7 Macrophages

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We investigated the effect of 17β-estradiol (E2) on the expression of daintain/AIF-1, a marker of activated macrophages, in RAW264.7. E2 upregulated the protein and mRNA levels of daintain/AIF-1 in similar manners under physiological concentrations of 10^{-11} M to 10^{-7} M. The application of ICI 182,780, an estrogen receptor (ER) antagonist, attenuated E2-induced daintain/AIF-1 production, suggesting the involvement of ER in this process.

**Key words:** 17β-estradiol (E2); daintain/AIF-1; upregulation; estrogen receptor (ER); RAW264.7 macrophages

It is well documented that estrogen, especially 17β-estradiol (E2), modulates cytokine gene expression. E2 exerts its effects via the intracellular estrogen receptor (ER). There are two subtypes of ER, ERα and ERβ, both of which are expressed in many immune cells.2,3) E2 inhibits inflammatory cytokine expression through ERα-dependent activation of the phosphatidylinositol 3-kinase (PI3K) pathway in lipopolysaccharide (LPS) activated-macrophages.4) These findings are consistent with significantly lower infection rates in women than in men.5) On the other hand, in several systems, E2 is reported to stimulate the production of proinflammatory cytokines such as IL-1, IL-6, IL-8, IFN-γ, and NO.10) These results may be responsible in part for the disproportionate susceptibility of females to autoimmune disease.11)

Allograft inflammatory factor-1 (AIF-1) was originally identified as a macrophage factor in heterotopic cardiac allografts in rats.12) Later, we isolated and characterized a polypeptide from porcine intestines and termed it “daintain.”13) It is probably identical with AIF-1. Hence we call the cytokine daintain/AIF-1. In humans, daintain/AIF-1 is a 143-amino acid, cytoplasmic, calcium-binding, inflammation-responsive scaffold protein. The daintain/AIF-1 gene maps to the major histocompatibility complex class III region on chromosome 6p21.3,14) which is known for clusters of genes involved in the immune response.15) Daintain/AIF-1 is constitutively expressed in monocytes and macrophages and is involved in macrophage activation.16) Although the detailed physiological functions of daintain/AIF-1 in vivo remain unclear, there is substantial evidence that its remarkable expression contributes to the pathogenesis of many autoimmune diseases, including rheumatoid arthritis,17) systemic sclerosis,18) experimental autoimmune neuritis,19) experimental autoimmune encephalitis and uveitis,20) insulin-dependent diabetes mellitus (IDDM),21) and plays roles in various inflammatory skin disorders.21) Therefore, daintain/AIF-1 is thought to play a fundamental role in monocyte and macrophage effector functions that contribute to inflammation and autoimmune reactions.

It has been found that E2 modulates cytokine expression, but no data are available regarding its regulation of daintain/AIF-1 expression. In the present study, we investigated the effects of E2 on the protein and mRNA levels of daintain/AIF-1 using RAW264.7 cells, a commonly used mouse macrophage cell line.

RAW264.7 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (FBS) at 37°C in a 5% CO2 humidified air atmosphere. Prior to the beginning of each experiment, cells were cultured in phenol red-free DMEM containing 10% dextran-charcoal treated FBS for at least 1 d. E2 and ICI 182,780 (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO). The DMSO final concentration in the culture medium was 0.1% or less. After incubation in serum-free medium for 6 h, the cells were treated with various concentrations of E2, ICI 182,780, or an equal volume of DMSO during the culture period.

For Western blot analysis, the cells were lysed in SDS-sample buffer (2% SDS, 62.5 mM Tris–HCl pH 6.8, 50 mM dithiothreitol, 10% glycerol, and 0.01% bromophenol blue), and boiled for 5 min. An amount of protein equal to each sample was separated by 15% SDS–PAGE, transferred onto a PVDF membrane (Millipore, Billerica, MA), and probed with primary rabbit polyclonal antibodies against daintain/AIF-1 (ProteinTech Group, Chicago, IL) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ProteinTech). Enhanced chemiluminescence was used to identify specific proteins (Pierce Biotechnology, Rockford, IL).

For RT-PCR analysis, total RNA was prepared from the cells using TRIzol-Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was reverse-transcribed from 2 μg of total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), according to the manufacturer’s recommendations. The gene-specific primers were as follows: daintain/AIF-1,

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**Abbreviations:** AP-1, activator protein 1; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; LPS, lipopolysaccharide.
Effect of E2 on Daintain/AIF-1 Expression in RAW264.7 Macrophages.

Cells were cultured at the indicated concentrations of E2 in serum-free media. A, The protein level of daintain/AIF-1 after treatment for 24 h was determined by Western blot. B, The mRNA level of daintain/AIF-1 after treatment for 6 h was determined by RT-PCR. C, Quantitative analysis of A and B. The density of the signals was measured using NIH image software, and was expressed relative to the control after normalization to the GAPDH signal. Data are the mean ± SD for three independent experiments. Statistical significance was analyzed by Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001 as compared with control).

It has been found that functional ERα, but not ERβ, is constitutively expressed in RAW264.7 cells.22,23 To investigate the involvement of ER in E2-dependent augmentation of daintain/AIF-1 expression, ICI 182,780, a “pure” estrogen antagonist, was tested at a 10^{-7} M excess concentration with respect to E2 on RAW264.7. As shown in Fig. 2, ICI 182,780 alone had no effect on daintain/AIF-1 expression. Co-treatment with ICI 182,780 attenuated the E2-mediated protein and mRNA expression of daintain/AIF-1, suggesting the involvement of ER in this process.

We postulated that E2 upregulates daintain/AIF-1 expression in RAW264.7 macrophages, and that this effect can be attenuated by ICI 182,780, an estrogen receptor antagonist. To our knowledge, this is the first evidence of E2 modulation of daintain/AIF-1 expression. It has been reported that E2 and LPS can synergize to augment daintain/AIF-1 expression, ICI 182,780, a ‘‘pure’’ estrogen antagonist, was tested at a 10^{-7} M excess concentration with respect to E2 on RAW264.7. As shown in Fig. 2, ICI 182,780 alone had no effect on daintain/AIF-1 expression. Co-treatment with ICI 182,780 attenuated the E2-mediated protein and mRNA expression of daintain/AIF-1, suggesting the involvement of ER in this process.

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several reports have mentioned that estrogen at physiological concentration exerts stimulatory effects on IL-1 in macrophage and monocyte populations.\(^5,6,23\) Given the proinflammatory effects of IFN-\(\gamma\), NO, and IL-1 in the immune response, these reports, as well as our data, suggest that E2 can modulate cytokine gene expression and contributes to the increased incidence of autoimmune disease in females.

Modulation by E2 of daintain/AIF-1 expression is due in part to changes in the transcription level, but the precise mechanism is unknown. It has been found that E2-bound ERs can bind directly to the estrogen response element (ERE) in the promoter of target genes and interact with other transcription factors such as activator protein 1 (AP-1) and specificity protein (Sp1), and regulate the transcription of related genes whose promoters do not include EREs.\(^28\) Sibinga et al.\(^28\) suggested that the promoter of daintain/AIF-1 contains multiple consensus binding sites for transcription factors, including AP-1, CCAAT/enhancer binding protein, and interferon regulatory factor (IRF-1). It is possible that E2 regulates daintain/AIF-1 transcription by binding to AP-1 responsive elements, but further studies are needed to determine the mechanism underlying the effect of E2 on daintain/AIF-1 expression.

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References