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Effect of Environmental Estrogens on IL-1β Promoter Activity in a Macrophage Cell Line

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Environmental estrogens or estrogen disrupters have recently received a great deal of attention because of their potential health impact on reproductive tissues. Few, if any, studies have been made on the impact of these compounds on the immune system. We sought to determine the activities of various environmental estrogens on the modulation of the interleukin-1β (IL-1β) gene in a model monocytic cell line, hER + IL-1β-CAT+. This cell line stably transfected with the human estrogen receptor, and an IL-1β promoter construct fused to the CAT reporter gene allows us to monitor the effect of estrogenic compounds on IL-1β promoter activity. 17β-Estradiol (E_2) markedly enhanced lipopolysaccharide- (LPS) induced IL-1β promoter-driven CAT activity in a dose-dependent manner. The mycotoxins α-zearalenol and zearalenone both exhibited full agonist activity, but at lower potencies, with EC_{50} values of 1.8 and 54 n*M*, respectively, compared with E_2 at 0.5 n*M*. In addition, genistein was a very low-potency agonist, having an EC_{50} of 1.5 μ*M*. Similar to the E_2 response, the slope factors for α-zearalenol, zearalenone, and genistein were close to 3.0, suggesting positive cooperativity in the estrogenic response. The activity of the mycotoxins appeared to be mediated through the estrogen receptor, since both the antiestrogens H1285 and ICI 182,780 effectively inhibited their agonist activity in a dose-dependent manner. Representative environmental estrogenic compounds both from plant and industrial sources were also tested. Unlike the mycoestrogens, none of the compounds, with the exception of genistein, synergized with LPS to enhance IL-1β promoter activity. When tested for antiestrogenic activity, the industrial compound 4-octylphenol was able to antagonize the response to E_2; however, the response was three orders of magnitude less potent than H1285. Naringenin, a plant flavonoid, showed little or no ability to antagonize the response to E_2. Overall, the results show that some environmental estrogens that display agonist activity in reproductive tissue also have an effect on IL-1 gene expression in hemopoietic-derived tissue.

Key Words: IL-1β; transcription; environmental estrogens; monocytes; cytokines; estrogen receptors.

Introduction

Estrogenic compounds are necessary for the development of the female reproductive tract and secondary sex structures, and are important for bone mineralization in females as well as males. In addition, estrogenic compounds have been shown to play roles in the cardiovascular and immune systems. Estrogens cause target tissue-specific responses through regulation of specific gene transcription. The generally accepted mechanism whereby estrogens allow for gene activation involves binding of the estrogen to the estrogen receptor and interaction of the receptor complex with estrogen-responsive elements (EREs) upstream of the targeted gene start site. However, some actions of estrogens appear to occur through alternative mechanisms, such as interaction with other transcription factors, such as *fos* and *jun*, or with nonclassical responsive elements.

Recently, a class of compounds loosely defined as environmental estrogens or estrogen disrupters has received a great deal of attention. These weakly estrogenic substances belong to classes of compounds as diverse as plant estrogens, fungal toxins, industrial pollutants, and plastic components. The health impact of these compounds has been hotly debated *(1,2)*, with most of the studies emphasizing impact on the reproductive system. Using various in vitro systems, several of these estrogen mimics have been shown to have differential sensitivities depending on the cell type and response measured *(3–5)*. We sought to determine the activities of various environmental estrogens on the modulation of the interleukin-1β (IL-1β) cytokine gene. Because the frequency and severity of autoimmune disorders are

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more prevalent in women, a study of the effect of various environmental estrogens on immune and inflammatory modulators is warranted.

We recently reported that the RAW264.7 cell line stably transfected with the human estrogen receptor (ER) and an IL-β promoter-CAT reporter construct showed an estrogen enhancement of lipopolysaccharide- (LPS) induced activity *(6)*. This response was estrogen-specific and ER-dependent. Interestingly, several estrogen metabolites displayed estrogen responses indistinguishable from estradiol. Thus, this present study investigates the activities of fungal toxins, phytoestrogens, and other estrogen mimics on cytokine production using this macrophage-cytokine model system.

Results

Because the biological effects of environmental estrogenic compounds have been studied primarily in breast cancer cell lines, we chose to examine a series of these compounds in a model monocytic cell line stably transfected with the human estrogen receptor (hER) and an IL-1β promoter-CAT reporter construct, hER + IL-1β-CAT+. This stably transfected cell line provides the opportunity to study the effect of various estrogenic/antiestrogenic compounds on IL-1β promoter activity under defined molecular conditions. We previously reported that estrogen markedly enhanced LPS-induced IL-1β promoter activity in this cell line *(6)*. Accordingly, we tested several potentially estrogenic compounds to determine if they were capable of enhancing LPS-induced activity comparable to E_2. As seen in Fig. 1, the EC_{50} for the E_2 response was 0.21 n*M* and reached a maximum at 0.5 n*M*. The slope factor for this response was 3.6, suggesting that positive cooperativity occurred for this response. α-Zearalenol exhibited full agonist activity, but was an order of magnitude less potent than E_2, having an EC_{50} of 1.8 n*M*. Zearalenone exhibited low potency, but full agonist activity, having an EC_{50} of 54 n*M*. Similar to the response to E_2, the slope factors for α-zearalenol and zearalenone were close to 3.0, suggesting positive cooperativity in the estrogenic response.

In order to determine if the response to zearalenone occurred via stimulation of the ER in the hER + IL-1β-CAT+ cells, two estrogen antagonists were tested for their ability to block the response. Zearalenone was tested at 100 n*M*, a concentration that stimulated CAT activity by about 90% of maximum. As shown in Fig. 2, H1285, a triphenylethylene antiestrogen similar to monohydroxy tamoxifen *(7)*, produced a dose-dependent antagonism, which was calculated to occur with an IC_{50} of 0.34 n*M*, assuming competitive antagonism prevails. This value is close to the IC_{50} calculated for competitive antagonism of the response to E_2 (*see* Fig. 3), indicating that the two agonists are both working through an ER to promote the response. ICI 182,780, similar to ICI 164,384 and considered a "pure" steroidal antiestrogen *(8)*, at 500 n*M* also fully antagonized the response to zearalenone. The inhibition by two different ER antagonists supports the contention that

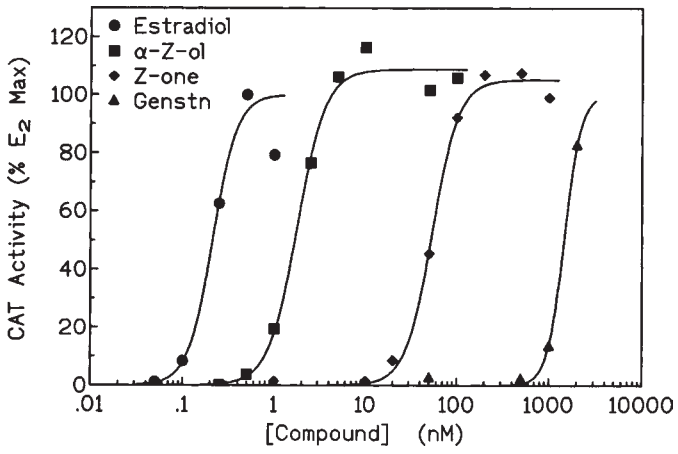

Fig. 1. Mycotoxins and the plant estrogenic compound genistein synergize to stimulate IL-1β promoter activity. The hER + IL-1β-CAT+ monocyte cell line stably transfected with both the hER and an IL-1β promoter CAT construct was exposed to increasing concentrations of E_2, α-zearanenol, zearalenone, or genistein + LPS at 50 ng/mL. CAT activity was always determined 24 h after LPS addition. E_2 as well as the various estrogenic compounds at the concentrations indicated were added to cultures 16 h prior to LPS or vehicle control. Data are reported as a percent of activity stimulated by 0.5 n*M* estradiol plus LPS. Representative data from one of two experiments is shown. Maximal CAT activity for E_2 + LPS (100%) as measured in cpm from the two experiments ranged from 13,080 to 69,096.

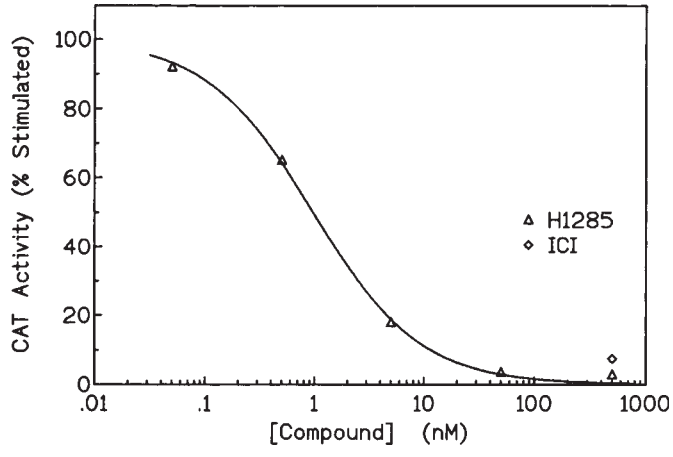

Fig. 2. The synergistic effect of zearalenone and LPS is inhibited by antiestrogens. hER + IL-1β-CAT+ cells were exposed to 100 n*M* zearalenone and the antiestrogens H1285 at the concentrations indicated and ICI 182,780 at 1000 n*M*, 16 h prior to LPS addition. CAT activity was assayed and determined as in Fig. 1 and were depicted as % CAT activity with the 100% value represented by the activity measured in the absence of antiestrogen. CAT activity as measured in cpm in the absence of antiestrogens ranged from 87,515 to 158,433 cpm in the H1285 and ICI 182,780 experiments, respectively. One of two separate representative experiments is shown.

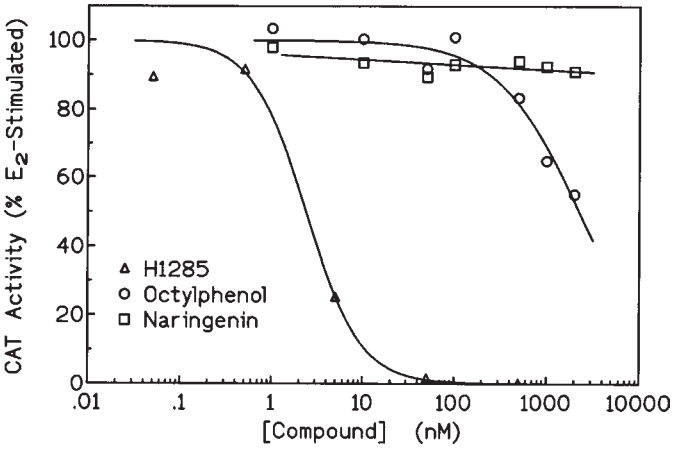

Fig. 3. The compounds octyphenol and naringenin display little or no antiestrogenic properties. The antiestrogenic properties of 4-octylphenol and naringenin are compared to H1285. hER + IL-1β-CAT+ was exposed to 0.5 n*M* estradiol plus varying concentration of H1285, octyphenol, or naringenin 16 h prior to LPS addition. Twenty-four hours after LPS stimulation, the cells were harvested and CAT activity determined as in Fig. 2; 100% values for E_2 in the absence of antiestrogen ranged from 119,160 to 150,315 cpm.

Table 1
Relative Activities of Plant and Industrial Estrogenic Compounds on IL-1β Promoter Activity

	Ligand	Conc., n*M*	% Maximal[a]
Plant	17 β-estradiol	0.5	100
	Naringen	2000	<0.001
	Naringenin	2000	0.5
	Luteolin	2000	3.6
	Genistein	2000	87.0
Industrial	Methoxychlor	2000	0.1
	4-Octylphenol	2000	<0.001
	bis-Phenol-A	500	0.03

[a]% Maximal CAT activity in hER + IL-1β-CAT+ cells stimulated by 50 ng of LPS in the presence of the listed estrogenic compounds. Addition of estrogenic compounds, LPS, and subsequent CAT assay was identical to assays performed in Fig. 1.

zearalenone promotes the response via an ER, although at a much lower potency than E_2.

Since α-zearalenol and zearalenone behaved as agonists, we next screened a variety of other known environmental estrogenic compounds both from plant and industrial sources. As seen in Table 1, none of the compounds, with the exception of genistein, syngerized with LPS to enhance IL-1β promoter activity. Genistein, however, displayed almost full agonist activity, but at a very low potency (EC_{50} of 1.5 μ*M*) (Fig. 1). Similar to the response of E_2, the slope factor of genistein was close to 3.0, suggesting positive cooperativity for the dose-related response.

Because a variety of the estrogenic compounds showed no agonist activity (Table 1), we next tested representative plant and industrial compounds for their abilities to function as antiestrogens. The response to maximally active E_2 (0.5 n*M*) was potently antagonized by the ER antagonist H1285 (Fig. 3). Assuming competitive antagonism, the IC_{50} for H1285 was calculated to be 0.71 n*M*. As seen in Fig. 3, 4-octylphenol was also able to antagonize the response to E_2; however, this compound was three orders of magnitude less potent than H1285. Naringenin showed little or no ability to antagonize the estrogenic response, even at concentrations as high as 2 μ*M*.

Discussion

Il-1β production is modulated in several tissues by the presence of sex steroids. For example, the loss of estrogen in postmenopausal women is thought to result in the elevation of several cytokines that promote bone degradation, which can result in osteoporosis *(9)*. The excessive or inappropriate production of IL-1 by E_2 *(10,11)* can potentiate and/or exacerbate autoimmune disease. In previous studies using the model RAW264.7 hER + IL-1β-CAT+ cell, we showed that E_2 dramatically amplified LPS-induced CAT activity in a dose-dependent fashion. This responsiveness was specific for estrogen and mediated by the stably introduced hER. Thus, these studies indicated that the enhancement of IL-1β production by E_2 *(6)* occurs at the transcriptional level. Interestingly, some metabolites of E_2 were equally as potent. For example, 17-epiestriol and 16-keto-17β-estradiol strongly stimulated promoter activity, whereas 4-hydroxyestradiol was less potent and 2-hydroxyestrone had no activity. This relative order of activities by E_2 metabolites was remarkably similar to that reported for the transforming growth factor-beta (TGF-β) cytokine promoter *(12)*. Because our system responded well to several E_2 metabolites, our present study was designed to test a variety of environmental estrogens to determine whether the hER + IL-1β-CAT+ monocytic cell line would also respond to these compounds, reported to have effects on reproductive tissue and breast cancer cell lines *(1,2)*.

Although there has been much interest in the role of environmental estrogens in the etiology of breast cancer over the past several years, little attention has been paid to the effect of such agents on the immune system, despite the fact that the incidence of most autoimmune diseases occurs in women. Previous epidemiological studies examining the effects of diets rich in various plant estrogens on the incidence of cancer have suggested that the low incidence of certain cancers in Eastern countries compared to Western countries may be related to diets rich in phytochemicals *(3,13,14)*. On the other hand, there has also been concern about the effects of environmental estrogens as promoters of breast cancer inasmuch as lifetime exposure to estrogens may be related to incidence of breast cancer *(4)*. Such interest has led to numerous studies of the interaction of various estrogenic and antiestrogenic agents with the ER and concomitant effects of a variety of estrogen-induced responses.

One class of compounds that has been of interest is the mycoestrogens. Reports of reproductive abnormalities in livestock that fed on contaminated grain led to the isolation of the zearalenols *(5–17)*. α-Zearalenol was discovered to be the naturally occurring diastereomer of zearalenol isolated from cultures of contaminated grain, such as corn, and has been reported to be more potent than zearalenone, also produced from *Fusarium (6)*. Studies of the affinity for the ER by several members of this family as well as biological activity both in vivo and in vitro have suggested that depending on the assay used, compounds with relatively high affinity may have weak biological activity owing to varying pharmacokinetic behaviors *(15)*. In our cultured model macrophage system, the mycoestrogens studied were clearly weaker than E_2, but nonetheless, were relatively potent agonists. To our knowledge this is the first report of the effect of mycoestrogens on cytokine gene expression. Whether these environmental estrogens as dietary components have any effect on cytokine gene expression in humans remains to be studied.

Plant flavonoids represent a class of phytoestrogens shown to have significant bioactivity in a variety of systems and assays. Naringenin, a compound found in grapefruit, was found by some investigators to be estrogenic *(3,4)*, whereas others have reported that it has significant antiestrogenic activity, both in the reproductive tract as well as in breast cancer cells *(18)*. In our studies, neither the flavanone naringenin nor the parent compound naringen had significant estrogenic activity; also, naringenin was not antiestrogenic. These results point to the differences in bioactivity of xenoestrogens, depending on the response being measured. We also demonstrated in our studies that genistein, an isoflavone found in soy products, was able to generate a near-maximal estrogenic response at pharmacological concentrations. Genistein has been reported to bind the ER *(3,4)* and elicit either estrogenic or antiestrogenic responses *(3)*, depending on the system/genes being studied. However, such studies are complicated by the fact that genistein has been reported to be an inhibitor of a variety of protein tyrosine kinases *(19)*. Nevertheless, experiments using other inhibitors of protein tyrosine kinases have shown that genistein can promote or inhibit estrogen-induced responses via binding to and activation of the ER *(20)*.

Other environmental disrupters, such as *bis*-phenol A, found in plastics and epoxy resins used in the lacquer lining of metal food cans, and 4-octylphenol, an industrial additive used in a wide variety of detergents, have been reported to have estrogenic activity in some assays *(5)*. In our study of estrogen stimulation of the IL-1β promoter, we found neither compound to be estrogenic; however, 4-octylphenol exhibited antiestrogenic activity at high concentrations.

Because there has been considerable controversy regarding the negative vs potentially beneficial effects of some of the various environmental estrogens *(1)*, and since there were no reports of the effect of such compounds on immune responses, our study was undertaken. Our results demonstrate that some, but not all, of the endocrine disrupters studied had effects in our model murine macrophage system, again suggesting that the effects of such agents are response-specific. In fact, we previously reported that many of the naturally occurring estradiol metabolites are far more potent and efficacious than the majority of estrogen mimics studied here. It may well be that the immune system is not as sensitive to environmental estrogens as the tissues and organs of the reproductive system. Because it is known that estrogens can modulate cytokine gene expression, it is important to monitor potential environmental estrogens for their effects on tissues that produce cytokines important in host defense mechanisms. Our model cell line, hER + IL-1β-CAT+, offers the opportunity to monitor estrogenic compounds rapidly for their ability to alter cytokine gene expression in at least one tissue of hemopoietic origin.

Material and Methods

Cell Culture and Cell Line

The hER + IL-1β-CAT+ cell line used throughout these experiments was maintained in Petri dishes in D5 media (Dulbecco's Modified Eagle's Medium containing 5% heat-inactivated fetal calf serum [HyClone, Logan, UT], 2 m*M* L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.75 mg/mL G418 [Sigma, St. Louis, MO], and 250 μg/mL hygromycin [Calbiochem, Cleveland, OH]). This stable cell line, which has been described *(6)*, was created by the initial introduction of the IL-1β promoter-CAT reporter construct, –4093 + I CAT *(21)*, along with a CMV-driven neomycin resistance vector into the murine monocyte cell line RAW 264.7 *(22)*. The reporter plasmid contains 4900 bp of IL-1β genomic DNA, which includes 4093 bp of upstream DNA, exon 1, the entire first intron, and the 5'-portion of the second exon, terminating immediately upstream of the translational initiation codon at position +807 (designated +I). We have consistently observed that a variety of IL-1β promoter CAT fusion genes require the presence of intron 1 for optimal CAT expression *(21)*. This stable reporter cell line was further transfected with the hER *(23)* and a hygromycin resistance plasmid.

The ER expression plasmid was the kind gift of Yoel Sadovsky (Washington University, St. Louis, MO) and has been described in detail *(23)*. The human ER was derived from the original cDNA clone of Chambon *(24)* and is driven by an SV40 enhancer and a metallothionein IIA promoter (hER).

Reagents

The compounds, 17β-estradiol, zearalenone, α-zearalenol, naringenin, naringen, genistein, luteolin, methoxychlor, and bisphenol A were purchased from Sigma Chemical Co., and 4-octylphenol was purchased from Aldrich (Milwaukee, WI).

Estrogen Enhancement Assay

The hER + IL-1β-CAT+ cells were seeded in 60-mm culture dishes at 4×10^6 cells/dish. After 3–5 h, vehicle, 17 β-estradiol (E_2), or the estrogenic compounds to be tested were added at various concentrations and the cells incubated overnight. The following day, LPS (50 ng/mL) was added, and the cultures harvested 24 h later to determine CAT activity. To perform the CAT assays, the harvested cells were washed with PBS, resuspended in 0.2 mL of 0.25 *M* Tris-HCl (pH 8.0), and lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation for 5 min at ~12,000*g* at 4°C in a microcentrifuge. The cellular supernatant was incubated at 60°C for 10 min, centrifuged as above, and 100 µL of each supernatant used to determine CAT activity, using [^{14}C]chloramphenicol (Amersham, Arlington Heights, IL) and an organic-phase extraction protocol *(25)*. Enhancement of IL-1β promoter activity was assessed by comparison of LPS-induced CAT activity in the presence and absence of E_2.

Data Analysis

CAT activity was determined as cpm converted. Data are reported as a percent of the activity stimulated by 0.5 n*M* estradiol plus LPS, after subtraction of background cpm (–LPS, –estrogen). Data from two individual experiments are shown as the average. Sigmoidal nonlinear regression analyses of the log dose–response relationships were determined using the computer program Inplot (Graphpad, San Diego, CA).

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