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Inhibitory effect of natural and environmental estrogens on thymic hormone production in thymus epithelial cell culture

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Abstract

The present study was carried out to assess the direct effect of natural estrogen and environmental estrogens on thymus epithelial cell (TEC) production/secretion of the thymic hormone thymosin-α 1 by using the technique of quantitative high-performance liquid chromatography. The presence of estrogen receptors in the TECs was also investigated. Murine TECs were cultured in the experimental DMEM medium containing various concentrations of natural or environmental estrogens, which was followed by determining the production of thymosin-α 1. The production of thymosin-α 1 by TECs was significantly inhibited by increasing concentrations of 17β-estradiol (natural estrogen) over 3×10^{-11} M, genistein (phytoestrogen) over 3×10^{-9} M, coumestrol (phytoestrogen) over 3×10^{-9} M, α-zearalanol (livestock anabolic) over 3×10^{-7} and bisphenol-A (plastic) over 3×10^{-6} M. Small amounts of estrogen receptor were present in the TECs. The above results clearly indicate that natural and environmental estrogens directly modulate TECs to produce thymic hormone probably through an estrogen receptor mechanism. Furthermore, our finding may be useful for evaluating biological effects of chemicals with estrogenic activity.

Keywords: Estrogen; Environmental estrogen; Endocrine disruptors; Thymus epithelial cells; Thymic hormone production

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1. Introduction

Natural estrogens such as 17β-estradiol, are well known to modulate immune responses in various animals including humans [1–4]. For example, changes in circulating estrogen levels during pregnancy deeply influence immune functions such as mitogen responses [5] and skin graft rejection times [6–7]. Fluctuations in circulating estrogen concentrations also affect thymic T-cell subpopulations [8]. In addition, thymus activity, in secreting its hormones, such as thymosins, thymulin, and thymostimulin, is markedly influenced by circulating estrogen levels [9–12]. In turn, thymosin(s), one of the amplifiers of T-cell immunity [13–15], stimulates estrogen secretion by the gonads through its direct action on the hypothalamus [16]. This hormone also stimulates the release not only of luteinizing hormone-releasing hormone (LHRH) from the superfused hypothalamus in vitro [17], but of luteinizing hormone (LH) from the pituitary after in vivo intra-cerebral administration [18]. In addition, estrogen manipulations have been found to affect both spontaneously occurring and experimentally induced autoimmune diseases such as systemic lupus erythematosus [19,20], lupus nephropathy [21] and autoimmune arthritis [22]. This evidence strongly supports the hypothesis that estrogen plays a strong role in the immune functions of many animals and humans.

Although this family of natural estrogens are steroidal in structure, a variety of exogenous non-steroids has been found to act like estrogens. In fact, estrogen-like chemicals (i.e., environmental estrogens), which are one of the endocrine disruptors, have been implicated in a number of human health disorders [23,24,25]. These substances are derived from a number of relatively common and abundant sources such as plants, plastics, agricultural products [26,27]. However, little is known about the pharmacological and/or toxicological effects on immunocompetent cells of exposure to these substances especially in reference to the cause of animal and human immune disorders. To address this issue, the present in vitro study focuses on the effects of natural and environmental estrogens on the production of thymic hormone by thymus epithelial cells, a type of immunocompetent cell, at its peptide levels.

2. Experimental procedures

2.1. Thymus epithelial cells

Thymus epithelial cells derived from rat thymus (TECs; IT-45R1) originally established by Itoh et al. [28] was used. This cell is known to produce thymic hormones [29] which induce the differentiation of prethymic progenitor cells into post-thymic cells through direct contact [30]. The presence of tonofilaments and cytokeratins within its cell body reveals this cell line to be of epithelial nature [28]. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM: Nipro, Tokyo), supplemented with 10% fetal calf serum (CDFCS: Bocknek, Ontario) pretreated with dextran-charcoal solution to remove endogenous estrogens, and then used in the experiments described below.

2.2. Chemicals

17β-estradiol; 1,3,5(10)-estratriene-3,17β-diol, genistein; 4′5,7-trihydroxyisoflavone (plant), α-zearalanol; 2,4-dihydroxy-6-(6α,10-dihydroxy-undecyl)benzoic acid μ-lactone (livestock anabolic), bisphenol-A; 4,4′-isopropyl-idennediphenol (plastic), progesterone; 4-pregnene-3, 20-dione, cholesterol; 3β-hydroxy-5-cholestene were purchased from Sigma Chemical Company (St Louis, MO). Coumestrol (3,9-dihydro-6H-benzofuro(3,2-c)-(1)benzopyran-6-one, plant) was obtained from Acros Organics (Geel, Belgium). Thymosin-α 1 standard was purchased from Sigma. All reagents were analytical grade.

2.3. Enzyme immunoassay of estrogen receptor (sandwich method)

Specific receptor for estrogen were assayed using an enzyme immunoassay kit (Abbot Laboratory, North Chicago, IL). The TECs were sonicated in a buffer consisting of 0.5 M KCl, 10 mM thioglycerol, 10 mM sodium molybdate, 10% (v/v) glycerol, and the sonicated sample was centrifuged at $800 \times g$ for 30 min at 4°C. The resulting supernatant was then used as a whole cell extract for enzyme immunoassay, according to the method described elsewhere [31]; briefly, the TEC extract was incubated with beads coated with rat monoclonal anti-estrogen receptor antibody at 4°C for 18 h. Each bead was then washed in phosphate buffered saline (pH 7.4). A second rat anti-estrogen receptor antibody, conjugated to horseradish peroxidase, was added to the beads, and they were further incubated for 1 h at 37°C. After adding *o*-phenylene diamine solution to the beads, a red color reaction was observed at 492 nm using a spectrophotometer (Abbot Laboratory). The concentration of estrogen receptors was determined by the standard curves run simultaneously with the sample specimen.

2.4. High-performance liquid chromatography (HPLC)

To determine the effect of the natural and environmental estrogens on thymosin-α 1 production, TECs were seeded in DMEM (phenol red-free) supplemented with 10% CDFCS in 250 cm^3 flasks. After two days of incubation, $3 \times 10^{-12} \sim 3 \times 10^{-4}$ M 17β-estradiol or various chemicals were added to the culture medium, and incubation was continued for 7 days. After 7 days of incubation, fresh DMEM transferred from each flask was mixed thoroughly with 5 volumes of 1N acetic acid, and then boiled in a hot-water bath. After thoroughly mixing with petroleum ether for 10 min, the mixture was centrifuged at $800 \times g$ for 20 min. The supernatant was filtered through Whatman No. 1 filter paper, and the filtrate evaporated on a hot-water bath. After adding Tris-HCl (pH 7.6) solution, the sample solution was applied to an Amicon molecular sieve column (Amicon Far East, Tokyo). Fractions constituting the void volume were lyophilized, dissolved in 10 mM HCl and chromatographed on a SynProPep C18 column (4.6 mm × 15 cm; The Separation Group, CA) in a Hitachi L-6210 HPLC system (Hitachi Electric., Tokyo). The linear gradient was programmed over 30 min from 100% solvent A (10 mM HCl) to 100% solvent B (30% acetonitrile–70% pure water). The peak flow was detected with a Hitachi L-400 UV Detector (Hitachi) at 215 nm, and the concentration of thymosin-α 1 determined from the standard curves which were run simultaneously with the sample specimens.

3. Results

3.1. Enzyme immunassay of estrogen receptor in the TECs

Substantial amount of estrogen receptors was detected in the supernatant of sonicated TECs; estrogen receptor concentration was 4.8 ± 1.1 fmol/mg protein. The receptor level compare well to other tissues; estrogen receptors in breast [32], endometrium [33] and thymus [34] were 4.19 fmol/mg, $120 \sim 322$ fmol/mg, 2.1 fmol/mg protein, respectively.

3.2. Inhibitory effect of natural and environmental estrogens on thymosin-α 1 production in TECs

The concentrations of thymosin-α 1 peptide in the supernatant from the culture flasks are shown in Table 1. Its concentration significantly decreased with increasing amounts of natural and environmental estrogens added to the culture media. In addition, the inhibition of thymosin-α 1 production was dose-dependent, a significant inhibition being obtained at 17β-estradiol doses over 3×10^{-11} M, genistein doses over 3×10^{-9} M, coumestrol doses over 3×10^{-9} M, α-zearalanol doses over 3×10^{-7} M and bisphenol-A doses over 3×10^{-6} M, respectively. No concentration of progesterone and cholesterol had a significant effect on the production of thymosin-α 1 (Table 1).

4. Discussion

The consequences of animal and/or human exposure to environmental estrogens (i.e., endocrine disruptors) on reproductive tissues have been well studied. However, little is known about the pharmacological and/or toxicological effects of such exposure on nonreproductive tissues, especially in reference to associations with immune disorders. To address this issue, we focused this study on whether environmental estrogens promote the immune function in a manner similar to natural estrogen.

There have been many papers reporting that the endocrine thymus produces a group of hormone-like peptides including thymosins [10,35], thymulin [9], and thymostimulin [11,12], and that these hormonal peptides all participate in the processes of immune functions by regulating the development of the thymus-dependent lymphoid system [35]. Although it is well known that adrenal steroids affect production and release of thymosins [36,37], few studies have been concerned with the relationship between the thymus and gonadal function [38]. To our knowledge the present in vitro findings are the first, even if partial, evidence to clearly demonstrate this functional relationship, namely, the action of natural and environmental estrogens on a defined cellular component within the thymic microenvironment.

Our previous in vivo studies have shown that estrogen receptor [39,40] and its mRNAs [40] are present in murine thymus tissues, and that estrogen receptor and its mRNA are both preferentially localized in the epithelial cells. Moreover, in other in vivo studies we have also reported that estrogen receptor-containing cells and the thymic hormone-producing cells are the same epithelial cells [39,41]. Based on those reports, the present in vitro experiment seems

Table 1
Effect of 17β-estradiol and environmental estrogens on thymosin-α 1 production in thymus epithelial cell culture[a]

Treatment ($n = 6$)	Thymosin-α 1 (ng/ml)	% Suppression (vs medium alone)
Medium alone	284 ± 22.6	–
+ 17β-estradiol		
3×10^{-12} M	272 ± 51.8	–
3×10^{-11} M	196 ± 32.7*	31
3×10^{-10} M	152 ± 16.3*	47
3×10^{-9} M	121 ± 15.8*	58
3×10^{-8} M	108 ± 19.2*	62
+ Genistein		
3×10^{-10} M	269 ± 34.8	–
3×10^{-9} M	206 ± 24.3*	28
3×10^{-8} M	174 ± 28.1*	39
3×10^{-7} M	132 ± 19.7*	54
3×10^{-6} M	112 ± 21.9*	61
+ Coumestrol		
3×10^{-10} M	291 ± 28.9	–
3×10^{-9} M	223 ± 19.4*	22
3×10^{-8} M	192 ± 20.1*	33
3×10^{-7} M	154 ± 18.8*	46
3×10^{-6} M	134 ± 21.9*	53
+ α-zearalanol		
3×10^{-8} M	278 ± 32.8	–
3×10^{-7} M	216 ± 11.7*	24
3×10^{-6} M	182 ± 21.7*	36
3×10^{-5} M	124 ± 19.1*	57
3×10^{-4} M	98 ± 27.7*	66
+ Bisphenol-A		
3×10^{-8} M	276 ± 27.4	–
3×10^{-7} M	274 ± 30.3	–
3×10^{-6} M	229 ± 24.6*	20
3×10^{-5} M	172 ± 23.7*	40
3×10^{-4} M	119 ± 17.5*	59
+ Progesterone		
3×10^{-12} M	282 ± 29.9	–
3×10^{-11} M	288 ± 32.7	–
3×10^{-10} M	289 ± 24.5	–
3×10^{-9} M	277 ± 38.6	–
3×10^{-8} M	264 ± 23.2	–
+ Cholesterol		
3×10^{-12} M	281 ± 42.6	–
3×10^{-11} M	284 ± 31.8	–
3×10^{-10} M	278 ± 20.7	–
3×10^{-9} M	276 ± 28.3	–
3×10^{-8} M	271 ± 32.5	–

[a] The mean percentage suppression of thymosin-α 1 production was calculated as (1−observed/expected) × 100, while 'expected' = medium alone (mean ng) and 'observed' = medium plus 17β-estradiol or environmental estrogens (mean ng).

* $P < 0.002$ vs medium alone control.

to add strong evidence that natural and environmental estrogens directly modulate TECs to produce the thymic hormone thymosin-α 1 through estrogen receptor.

In conclusion, the present results showed that natural and environmental estrogens suppress the production of thymic hormone, and that all environmental estrogens derived from plants, plastic and livestock anabolic have this action although to varying degrees. The effect was seen with $3 \times 10^{-9} \sim 3 \times 10^{-6}$ M as a significant exposure level, which is rarely present in the environment. Thus, these environmental estrogens cannot be regarded as risk factors for immune disorders ordinary conditions or ordinary living conditions; however, Olea and Olea-Serrano [42] recently demonstrated that environmental estrogens were present in both extracted foods and water from autoclaved cans at concentration of μg ~ mg level per can, for example. Thus, the results of present study suggest that we must recognize the possibility that environmental estrogens can suppress the production of thymic hormones such as thymosin-α 1.

Finally, the precise mechanism of environmental estrogen action is still unclear. Further efforts to resolve this question are underway in our laboratory.

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