

Regulation of Peroxisome Proliferator-Activated Receptor- γ Expression in Human Eosinophils by Estradiol

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Key Words

Asthma · Eosinophils · Estradiol · PPAR γ · Sex difference

Abstract

Background: Peroxisome proliferator-activated receptor- γ (PPAR γ) is a nuclear receptor that regulates not only adipogenesis but also immune reaction. We previously demonstrated that human eosinophils expressed functional PPAR γ , although the modulator of PPAR γ expression is less well understood. Because clinical studies have shown that the efficacy of PPAR γ agonists as insulin sensitizers is stronger in women than in men, we investigated whether sex hormones caused any changes in eosinophil PPAR γ expression levels.

Methods: First, purified human peripheral blood eosinophils were cultured with 17 β -estradiol for 24 h, followed by PPAR γ measurement using a flow cytometer. Next, eosinophil PPAR γ expression and serum estradiol were studied in 10 healthy women during the menstrual and follicular phases to identify the physiological significance of estradiol. Eosinophil PPAR γ expression was also compared in 22 men, 21 non-pregnant women, and 15 pregnant women. **Results:** We observed that PPAR γ protein expression in eosinophils was significantly enhanced by 10⁻⁶ M 17 β -estradiol. Although serum estradiol concentration was increased during the follicular phase, PPAR γ expression levels were not affected by the menstrual cycle. In addition, no significant differences in PPAR γ expression were observed in terms of sex

and pregnancy. **Conclusions:** These findings suggest that estradiol potentially upregulates eosinophil PPAR γ expression in vitro, although some other mechanisms might be involved in its regulation in vivo.

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Introduction

The peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of the ligand-activated transcription factors. PPARs promote the transcription of target genes by forming heterodimers with retinoid X receptor (RXR) and binding to specific motifs known as PPAR-responsive elements (PPRE) [1]. To date, three mammalian PPAR subtypes that are encoded by separate genes have been identified- α , β (also termed δ), and γ . Among them, high levels of PPAR γ are expressed in adipocytes, and it plays a key role in regulating insulin sensitization and adipogenesis [2].

Multiple lines of evidence have shown that PPAR γ is also expressed by human hemopoietic cells and serves as a negative regulator in the immune system [3]. Human eosinophils, the major effector cells in the pathogenesis of allergic inflammation, express PPAR γ , RXR α , and RXR γ [4, 5]. We previously demonstrated that stimulation of eosinophils with a synthetic PPAR γ agonist inhib-

ited factor-induced eosinophil activation [5–7]. In addition, it has been reported that administration of PPAR γ agonists or adenoviruses carrying PPAR γ cDNA had beneficial effects on pathological conditions, including airway hyperresponsiveness and lung eosinophilia, in a murine model of asthma [8–10]. Thus, the evidence suggests that targeting PPAR γ may be a new therapeutic modality for the treatment of asthma [11, 12].

With the ability to directly bind PPAR γ , thiazolidinediones are clinically used as insulin-sensitizing drugs. Interestingly, clinical studies have shown that the same dose of thiazolidinedione (pioglitazone) is more effective in women than in men [13]. In addition, Vidal-Puig et al. [14] found sexually dimorphic expression with increased expression of PPAR γ mRNAs in the subcutaneous adipose tissue of women compared with men. In this study, we investigated the effect of estradiol and differences in PPAR γ expression according to menstrual cycle, sex, and pregnancy to clarify whether sex hormones are physiological modulators of PPAR γ expression in human eosinophils.

Subjects and Methods

Subjects

To study the effect of estradiol on eosinophils *in vitro*, peripheral venous blood was obtained from non-pregnant, medication-free subjects with mild to moderate eosinophilia. The eosinophils were further purified from whole blood as described below. Informed consent was obtained from each subject, and the study protocols were approved by the Ethics Committee of Akita University School of Medicine. To study the influence of the menstrual cycle on PPAR γ expression, peripheral blood was obtained during the follicular and menstrual phases from 10 regularly cycling healthy women (mean age 34.6) who kept daily body temperature records. To study the sex difference, peripheral blood samples were obtained from 22 men and 21 non-pregnant women. They were healthy medical students who visited our hospital for a routine check-up during the same period. Blood samples were also obtained from 15 pregnant women who visited our hospital for a check-up or were admitted for medical care or delivery. They were at 26–41 (mean 34.5) weeks of gestation. Five had pregnancy hypertension, 4 were at threatened premature delivery, and 6 had an abnormal amount of amniotic fluid. No subjects received systemic corticosteroid or immunosuppressive drugs.

Cell Preparation

Eosinophils were isolated from peripheral blood by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients, as previously reported [5]. The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The eosinophils (>98% purity) were then suspended in Hank's balanced salt solution with 1% fetal calf serum in tubes coated with 3% hu-

man serum albumin. The purified eosinophils were resuspended at a density of 1×10^6 cells/ml in RPMI 1640 (Gibco, Grand Island, N.Y., USA) medium supplemented with 10% fetal calf serum and incubated with the indicated concentrations of 17 β -estradiol (Sigma Chemicals, St. Louis, Mo., USA) for 24 h at 37°C. RPMI 1640 medium was supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml).

Flow Cytometric Analysis

Flow cytometry was used for studying the protein expression of PPAR γ as previously described [15, 16]. Purified eosinophils were washed twice with PBS and then permeabilized with FIX & PERM (Caltag Lab., San Francisco, Calif., USA). Cells were stained with 1:500 diluted rabbit polyclonal anti-PPAR γ antibody (Biomol Research Laboratories Inc., Plymouth Meeting, Pa., USA) and phycoerythrin-conjugated, affinity-purified anti-rabbit IgG (Rockland, Inc., Gilbertsville, Pa., USA). Rabbit IgG Ab (R&D, Minneapolis, Minn., USA) was used as an isotype-matched control. The stained cells were analyzed using a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA). PPAR γ expression was assessed as the ratio of the mean fluorescence intensity (MFI) of the sample and the isotype-matched control. Some results were expressed using the percentage of stimulated cells' MFI ratio to that of non-stimulated cells (% MFI ratio). Alternatively, donor variation in eosinophil PPAR γ expression was measured by modified whole-blood analysis [17]. In brief, heparinized whole blood was hemolysed using CAL-lyse (Caltag Lab.), according to the manufacturer's instructions. Cells were then permeabilized and stained in a similar way with purified eosinophils. The MFI of each sample was obtained in the area representing eosinophils on the cytogram.

Serum Estradiol Measurement

Fresh-frozen serum samples from healthy and pregnant women were thawed and assayed for estradiol on an enzyme immunoassay analyzer AIA-21 (Tosoh Corp., Tokyo, Japan).

Statistical Analysis

The Excel Statistics 2006 package (SSRI Ltd. Co., Tokyo, Japan) was used for statistical analysis. The results were expressed as the mean $\pm$ SD. The data of two groups were compared using paired t test. For the comparison between groups, analysis of variance (ANOVA) was used. If the ANOVA was significant, post-hoc pairwise comparisons were conducted using Dunnett's test. Significance was established at the $p < 0.05$ level.

Results

From the standpoint of eliminating interactive systemic effects, an *in vitro* study is essential to understand the role of estrogen in modulation of eosinophil PPAR γ expression. We first studied the effect of 17 β -estradiol on PPAR γ protein levels using a flow cytometer. In line with our previous report, purified fresh eosinophils constitutively expressed PPAR γ [5, 16, 17]. Eosinophils were cultured with various concentrations of 17 β -estradiol for

24 h, followed by measurement of PPAR γ expression (fig. 1). We found that PPAR γ expression was increased concentration-dependently by 17 β -estradiol. The maximal effect was observed at 10^{-6} M, which is the highest concentration we tested, and PPAR γ expression increased by $116.5 \pm 18.3\%$. In all cases, we observed no cytotoxic effects of 17 β -estradiol that were confirmed by trypan blue dye exclusion assay.

We next determined whether estrogen is a physiological upregulator of PPAR γ expression *in vivo*. Estrous cycle-related changes in PPAR γ expression in parametrial white adipose tissues from female mice have been reported [18]. Thus, blood was obtained during the follicular and menstrual phases from 10 healthy non-pregnant women to study the influence of women's menstrual cycle. As we expected, serum estradiol concentrations were significantly higher during the follicular phase (range 60.9–321.5, mean 147.2 pg/ml) compared with the menstrual phase (range 25.0–94.1, mean 52.8 pg/ml). However, eosinophil PPAR γ expression did not differ between the two phases (mean of MFI ratio $\pm$ SD was 1.57 ± 0.22 and 1.62 ± 0.26 , respectively) (fig. 2), and there was no correlation between estradiol and PPAR γ expression levels (data not shown). We next compared eosinophil PPAR γ expression levels of 22 men and 21 women. However, as shown in figure 3, no significant differences were observed (men 1.49 ± 0.29 , women 1.50 ± 0.37). It has been known that estrogen levels increase dramatically during pregnancy; the plasma level increases from 10^{-11} in the normal phase to 3×10^{-7} M by the end of pregnancy [19]. Thus, we also measured the eosinophil PPAR γ expression levels from 15 pregnant women, although higher expression was not observed compared to men or non-pregnant women (1.44 ± 0.41) (fig. 3). Among the pregnant women, we could measure serum estradiol concentrations in 11 subjects. Although we confirmed a significant increase in estradiol (range 8,528.9–44,553.0, mean 19,977.1 pg/ml), there was no correlation between estradiol and PPAR γ expression levels (data not shown).

Discussion

Most allergic patients who require medical treatment have been sensitized against allergens and developing inflammatory reactions. Thus, strategies that modulate effector phases of allergic response have clear potential to provide a therapeutic benefit [20]. Eosinophils that accumulate at an allergic inflammatory site contribute to tissue injury, vascular leakage, mucus secretion, and airway

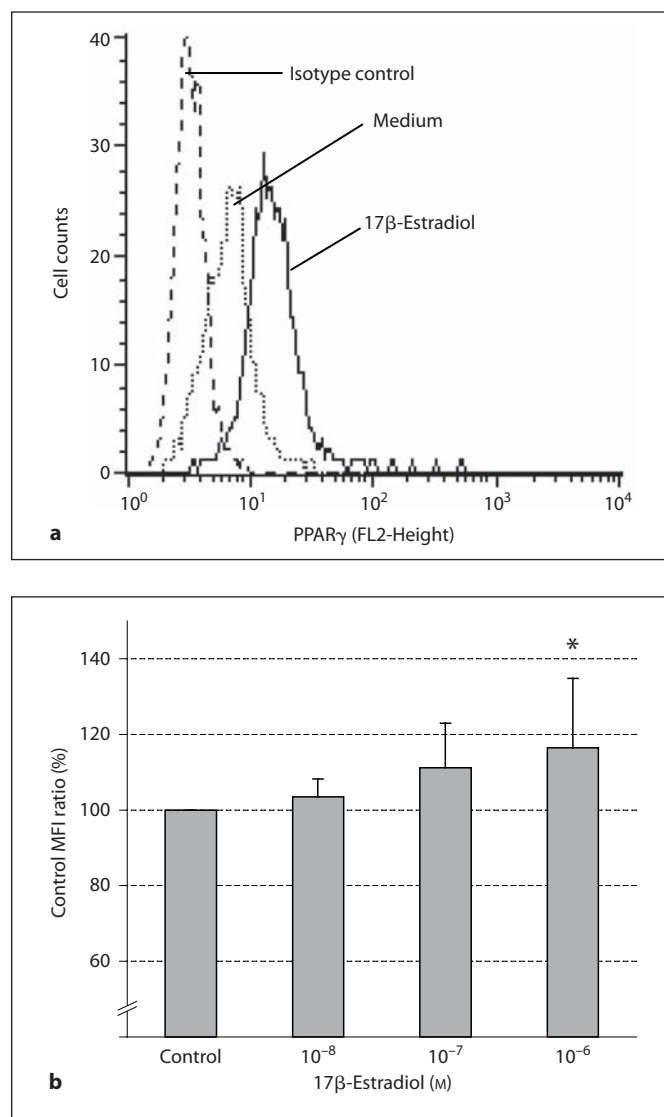
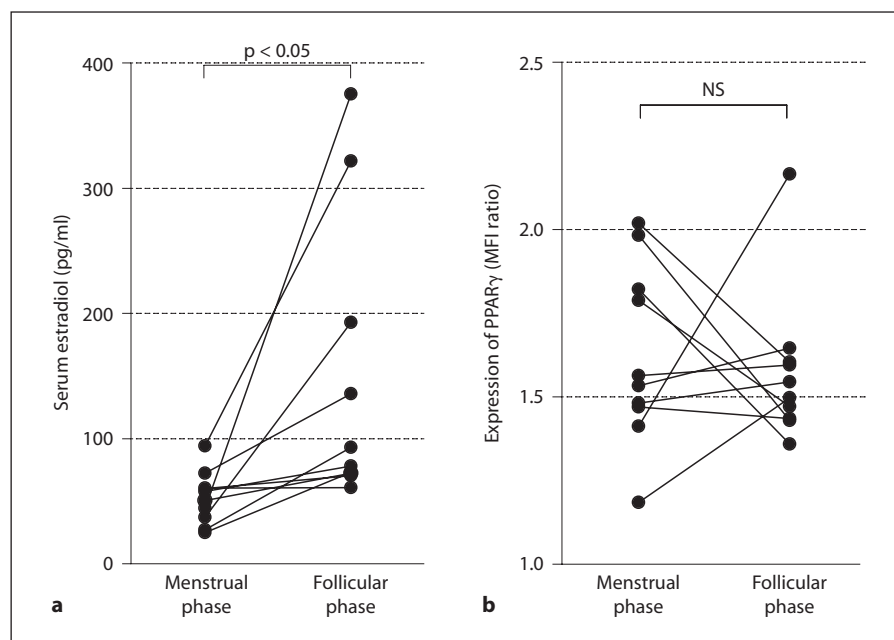


Fig. 1. Effect of estradiol on PPAR γ expression. Purified eosinophils were incubated with 17 β -estradiol for 24 h. PPAR γ expression was then measured using a flow cytometer. **a** Histograms showing the typical results of PPAR γ expression in purified eosinophils incubated with the medium or 10^{-6} M 17 β -estradiol. Histogram of isotype-matched control did not change with stimulation with 17 β -estradiol (not shown). **b** The concentration response of estradiol on PPAR γ expression in purified eosinophils. The results are expressed as relative protein levels with a value of 100% for the respective controls. Data are expressed as the mean of 6–7 experiments (eosinophils) $\pm$ SD. * $p < 0.05$.

contraction by releasing cytotoxic granule proteins, reactive oxygen species, and lipid mediators [21]. We have shown that therapeutic concentrations of a PPAR γ agonist attenuate cytokine/chemokine-induced eosinophil functions such as chemotaxis [5, 11], survival [5], degran-

Fig. 2. Serum levels of estradiol (**a**) and eosinophil PPAR γ expression (**b**) in 10 women at the follicular and menstrual phases. Whole blood was obtained during the indicated menstrual cycle, and the serum concentration and eosinophil PPAR γ expression were measured as described in 'Materials and Methods'. Although serum estradiol significantly increased during the follicular phase, PPAR γ expression did not differ between the 2 phases. NS = Not significant.



ulation [6], and adhesion [7]. Currently, exogenous PPAR γ agonists are suggested to be a potential therapy for human asthma since the clinical safety of PPAR γ agonists has been demonstrated, and their effectiveness in an animal model of asthma was similar to that of glucocorticoids [22]. In addition, several studies have shown that adenoviral delivery of a PPAR γ cDNA expression plasmid reduced the pathophysiological symptoms in murine models of asthma [10, 22]. Thus, artificial overexpression of PPAR γ could be another therapeutic modality for the treatment of allergic inflammatory diseases, and to understand the physiological regulation of PPAR γ in allergic inflammatory cells is potentially important. In this study, we reported for the first time that 17 β -estradiol upregulated the expression of PPAR γ protein in human eosinophils in vitro.

In line with our observation, Ibabe et al. [23] reported that PPAR γ expression levels in zebrafish hepatocytes was increased by culturing with 17 β -estradiol for 24 h. Like PPAR, the estrogen receptor (ER) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. Although the influence of estradiol on cell function was previously assigned exclusively to genomic reaction, it is known that estradiol activates the membrane form of ER and can rapidly induce intracellular cyclic AMP (cAMP) elevation [24]. Recently, we found that the bronchodilators theophylline and procaterol upregulated PPAR γ mRNA and protein expression in eosinophils [15, 16]. Because the functions of these

drugs are mediated through cAMP signaling, elevated cAMP in eosinophils might play a role in augmentation of PPAR γ expression. The importance of cAMP in PPAR γ modulation was supported by previous findings [25], and increased cAMP stimulates expression of CCAAT/enhancer-binding proteins (C/EBPs), which play a crucial role in enhancement of PPAR γ expression [26].

Although eosinophil PPAR γ expression was upregulated by stimulation with estradiol in vitro, we could not find any difference in eosinophil PPAR γ expression from different donors in terms of sex, menstrual cycle, and pregnancy. Particularly at late pregnancy, serum concentrations of estradiol increase up to 100- to 1,000-fold from the normal state and can exceed 10^{-7} M. In the present study, stimulation of eosinophils with 10^{-7} M estradiol increased PPAR γ expression, although it was not statistically significant. Therefore, one explanation is that their serum estrogen levels were not sufficient to directly trigger the rise of eosinophil PPAR γ expression. Another limitation of this study was complications in some pregnant women such as hypertension or an abnormal amount of amniotic fluid, although their influence on PPAR γ expression in immune cells has not been studied.

Several recent studies using in vivo models have investigated the regulation of PPAR γ expression by administration of estradiol or ovariectomy. Contradictory to our study, estradiol decreased mRNA and/or protein expression of PPAR γ in rat pituitary [27] and uterus [28], and also human adipose tissue [29]. Thus, the effect of estra-

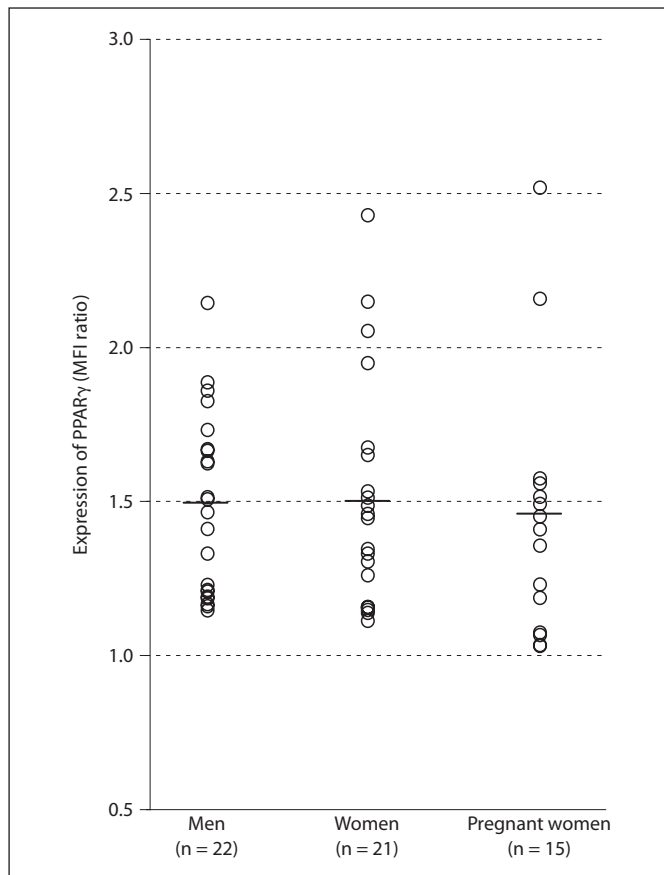


Fig. 3. PPAR γ expression levels in peripheral blood eosinophils. Whole blood was obtained from 22 men, 21 women, and 15 pregnant women, and PPAR γ expression was measured using a flow cytometer. There was no statistical difference between the 3 groups. Each bar shows the mean MFI ratio.

diol might be tissue type dependent, or some other factor(s) regulated by estradiol might be involved in downregulation of PPAR γ expression. Interestingly, Ibabe et al. [23] reported the contradictory result that estradiol-stimulated hepatocytes increased the levels of PPAR γ per cell, whereas the numbers of PPAR γ -positive cells were decreased. Thus, further studies both in vivo and in vitro are necessary to shed light on the physiological effect of estrogen on PPAR γ expression.

Previously reported changes in PPAR γ expression in allergic disease are controversial. Using immunofluorescence staining of bronchial biopsy samples obtained from asthmatic patients, Benayoun et al. [30] reported that one of the PPAR γ -expressing cell types in bronchial submucosa were eosinophils. In addition, as compared to that from normal subjects, PPAR γ expression was upregulated in asthmatics who did not receive corticosteroid treat-

ment. Conversely, Kobayashi et al. [31] reported that mRNA expression of PPAR γ in bronchoalveolar lavage cells was decreased after segmental allergen challenge to asthmatics. With regard to allergic rhinitis, one study reported that the mRNA expression of PPAR γ in nasal mucosa did not differ from that of normal subjects [32], but another reported the upregulation of PPAR γ in allergic patients [33]. Concerning eosinophils, Woerly et al. [8] reported that PPAR γ expression levels assessed by flow cytometer had no correlation with the activation marker CD25 or eosinophilic cationic proteins in serum. Consistent with this, we previously reported that eosinophil PPAR γ expression did not change as a result of in vitro stimulation with IL-5, a major eosinophil-activating cytokine produced abundantly in allergic inflammatory sites [5, 15]. The modulation of PPAR γ expression in allergic inflammation should be investigated further. Such studies will provide interesting insights into the physiological importance of PPAR γ in the immune system.

In conclusion, the present study indicates the existence of cross-talk between estrogen and PPAR γ because estradiol upregulates PPAR γ expression in human eosinophils in vitro. Our findings also suggest that PPAR γ expression in circulating eosinophils is not regulated by serum estradiol, but further studies are required to verify the in vivo modulation of PPAR γ expression.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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