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ORIGINAL RESEARCH ARTICLES

Functional Characterization of Exonic Variants of the *PPARGC1B* Gene in Coregulation of Estrogen Receptor Alpha

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Peroxisome proliferator-activated receptor gamma coactivator 1 beta (*PPARGC1B)* is a coactivator of estrogen receptor (ER)α and ERβ. We previously demonstrated a significant association between a variant of exon 5 of the PPARGC1B gene (+102525 G>A, *R265Q*) and airway hyperreactivity (AHR). The aims of the study were to evaluate the genetic effects of variants of the *PPARGC1B* gene on the function of ERs. *PPARGC1B +102525G and A* gene constructs were generated using PCR and cloned into a pCMV4 promoter vector. A luciferase reporter assay was undertaken in 293T cells cotransfected with one of the *PPARGC1B +102525G>A* constructs, ERα, and an estrogen response element (ERE) containing a luciferase construct after treatment with 17β-estradiol. According to the luciferase reporter assay, the *+102525A* allele showed higher ERα activity than the *+102525G* allele in response to stimulation with 17β-estradiol. In addition, the interaction between *ER*α and *PPARGC1B* was evaluated by coprecipitation assay. Human influenza hemagglutinin-tagged *PPARGC1B* coprecipitated more intensely with *ER*α in the +102525A than the +102525G construct after 17β estradiol treatment. The variant *+102525A* allele enhances the activity of ERα to a greater degree than the *+102525G* allele of *PPARGC1B*.

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Introduction

Noteworthy sex differences have been demonstrated with respect to the prevalence and severity of asthma. Asthma is more frequent in females after than before menarche, and the incidence of asthma is higher in reproductive-age females than males (Strachan *et al.*, 1996; Wjst and Dold, 1997). A total of 25–40% of reproductive female subjects experience premenstrual worsening of asthma symptoms, possibly induced by an increase in airway inflammation caused by changes in levels of female hormones (Eliasson *et al.*, 1986; Oguzulgen *et al.*, 2002; Vrieze *et al.*, 2003). About 20% of asthmatic females experience exacerbation around the late second trimester of pregnancy (Murphy *et al.*, 2006).

In addition, postmenopausal females had a significantly lower risk of asthma than premenopausal females, and postmenopausal use of estrogen has been associated with an increased rate of newly diagnosed asthma in menopausal females (Troisi *et al.*, 1995; Romieu *et al.*, 2010). These data indicate that changing levels of estrogen and progesterone may play a role in the development and exacerbation of asthma in some but not all female asthmatics.

Estrogens exert their effects through interactions with two intracellular receptors: estrogen receptor (ER)α and ERβ (Mangelsdorf *et al.*, 1995). With the help of coregulators, these receptors act as potent regulators of gene transcription (Glass *et al.*, 1997). Peroxisome proliferator-activated receptor gamma coactivator 1 beta (*PPARGC1B*) is a coactivator for various intracellular receptors, including ERs, peroxisome proliferator-activated receptor (*PPAR*), and glucocorticoid receptors (GRs) (Kressler *et al.*, 2002).

The product of the *PPARGC1B* gene is involved in fatty acid oxidation, mitochondrial biogenesis, and muscle fiber formation (St-Pierre *et al.*, 2003; Arany *et al.*, 2007; Ling *et al.*, 2007), malfunctions which are associated with impaired muscular endurance, metabolic syndrome, and type 2 diabetes (Park *et al.*, 2006; Ahmetov *et al.*, 2009; Delgado-Lista *et al.*, 2014). At the genetic level, single-nucleotide polymorphisms (SNPs) of the *PPARGC1B* gene are associated with the risk of ER-positive breast cancer due to their interactions with the ER (Li *et al.*, 2011).

We previously reported strong associations of two SNPs of *PPARGC1B* with airway hyperreactivity (AHR), as determined from the concentration of methacholine required to induce a 20% decline in FEV1 (PC20) (Lee *et al.*, 2011):

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–427C>T in the promoter and *+102525G>A* in the coding region ($P = 0.005–0.0004$). The *+102525G>A* SNP, which is located in codon 265 of *PPARGC1B* in exon 5, induces an amino acid substitution of arginine to glutamine (R265Q). This amino acid substitution is expected to induce structural or functional changes in the PPARGC1B protein, because arginine is the most hydrophilic amino acid (hydrophobicity: 0.00) and is frequently present in the active and catalytic sites of proteins. In contrast, glutamine is less hydrophilic than arginine (hydrophobicity: 0.43) and is found mainly in functional loops and regions, as determined using Amino Acid Explorer (www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi).

The PPARGC1B protein is known to interact with *ER* by binding to estrogen response elements (ERE) on DNA through its two LXXLL motifs, nuclear receptor (NR)1 and NR2 (Kressler *et al.*, 2002). NR1 is missing in one isoform of *PPARGC1B* lacking 39 amino acids (positions 156 to 194, encoded by exon 4). NR2 is located at amino acids 293–300, encoded by exon 5. R265Q is located in exon 5, 71 amino acids from NR1 and 28 from NR2. Therefore, the substitution caused by *+102525G>A* may alter the coactivator function of *PPARGC1B* and lead to a functional change in the activity of estrogens. However, functional differences in the two proteins have not been evaluated with respect to the biologic effects of estrogen and their relationship to AHR, which was reported by us previously (Lee *et al.*, 2011). In the present study, we investigated the functional effects of the amino acid substitution caused by *+102525G>A* on ER coactivation using *in vitro* ER- and ERE-transfected cell lines.

Materials and Methods

Plasmid constructs

To generate the *+102525G* construct, a point mutation was induced. Briefly, the pCMV4/PPARGC1B vector (OriGene, Rockville, MD)-containing *PPARGC1B+102525A* was PCR amplified using a forward primer containing a *Xho*I restriction enzyme site and a G allele-containing reverse primer adjacent to *+102525*. A second PCR fragment was amplified using a G allele-containing forward primer adjacent to *+102525* and a reverse primer containing an *EcoR*I restriction enzyme site. Overlap PCR was performed with the first and second PCR constructs using a forward primer containing an *Xho*I restriction site and a reverse primer containing an *EcoR*I restriction site. Primer sequences are presented in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/dna). The amplified PCR product was cloned into *Xho*I and *EcoR*I sites in the pCMV4 vector (OriGene) and designated pCMV4/*PPARGC1B +102525G*.

Plasmids were purified using the QIAGEN Plasmid Maxi Kit (QIAGEN GmbH, Hilden, Germany). At each step, the sequences were confirmed by direct sequencing. To measure the activity of PPARGC1B, the construct was cotransfected with the expression plasmid pCMV6/ESR1 (ER 1; ERα), a full-length cDNA clone (OriGene), and an estrogen response element (ERE)-containing luciferase reporter plasmid (pERE-luc; Panomics, Beijing, China).

For the coprecipitation assay, the full-length cDNA of PPARGC1B was transferred from the pCMV4 vector into the *Xho*I and *EcoR*I sites of the EGFPN1 vector (EGFPN1-HA tag), which contains a tag with a human influenza hemagglutinin (HA) epitope (5′-ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT-3′) (Sino Biological, Inc., Beijing, China).

Cell culture and transfection

293T cells (ATCC; CRL-11268) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA) containing L-glutamine (2 mM), HEPES (25 mM), 9% heat-inactivated FBS (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 ng/mL streptomycin (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2. Charcoal-stripped fetal bovine serum and phenol red-free media were used to assay hormone responses. Twenty-four hours before transfection, 5×10^5 cells in a 2 mL volume were seeded in six-well plates without antibiotics to 90–95% confluence at the time of transfection. The 293T cells were transiently transfected with pCMV4/PPARGC1B using Lipofectamine 2000 (Invitrogen) with a DNA (μg): Lipofectamine (μL) ratio of 1:2 in Opti-MEM (Invitrogen). One microgram of pRL-TK control vector (Promega, Madison, WI) was co-transfected for normalization of Renilla luciferase activity.

The amounts of expression and reporter plasmids used in coactivation assays were as follows: 1 μg pCMV6/ESR1, 1 μg pERE-luc, and 2 μg pCMV4/*PPARGC1B +102525A* or *+102525G*. After 6 h of transfection, the cells were washed and incubated for a further 24 h in fresh medium containing 17β estradiol (E4389; Sigma-Aldrich Co. LLC., St. Louis, MO), progesterone (p8783; Sigma-Aldrich), dexamethasone (D2915; Sigma-Aldrich), or vehicle (0.1% ethanol or Me_2SO). After 48 h of transfection, the cultured cells were washed twice with phosphate-buffered saline (PBS) and lyzed by mechanical scraping with 400 μL of Reporter Lysis Buffer (Promega). After centrifugation for 2 min at 12,000 rpm, firefly luciferase values in the supernatant were measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to those of Renilla luciferase.

Coimmunoprecipitation

For coimmunoprecipitation (co-IP) of FLAG-ERα protein with HA-PPARGC1B *265R* or 265Q protein, 20 μg of nuclear protein extract were incubated in the presence of 0.1% Triton X-100 with 3 μL of monoclonal mouse anti-FLAG antibody (Sigma-Aldrich) or 15 μL anti-FLAG agarose (Sigma-Aldrich) with rotation overnight at 4°C. The anti-FLAG antibody was captured using protein A agarose beads for 2 h at 4°C. Beads were collected by centrifugation and washed four times using lysis buffer supplemented with 1% Triton X-100 and 150 mM NaCl. The collected immunoprecipitate was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and HA-PPARGC1B was detected using a horseradish peroxidase-conjugated mouse monoclonal anti-HA antibody (Abcam, Cambridge, United Kingdom).

Results

Effect of PPARGC1B+102525G>A (R>Q) *on estrogen activity* in vitro

The 293T cell line was selected for the following experiments because the cells expressed little amount of internal ERα mRNA and PPARGC1B protein (data not shown). To

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investigate whether the *+102525G>A* SNP affects the ERα coactivating function of *PPARGC1B*, the activities of the *PPARGC1B+102525G* and *A* variants were measured in 17β estradiol-treated 293T transfectant cells. The normal range of estradiol in the circulating blood is from 0.01 to 1 nM, which increases up to 10 nM in early pregnancy (Speroff and Fritz, 2004). Regarding the physiological concentration, we used 0.5, 5, and 50 nM of 17β estradiol for ERα stimulation.

First, luciferase reporter activity was measured in 293T cells cotransfected with the pERE-luc and pCMV6/ESR1 constructs after 17β estradiol treatment (Fig. 1). Luciferase activity was increased by the estradiol treatment in a dose-dependent manner (6.28 ± 0.33-fold increase in unstimulated cells vs. 16.16 ± 0.58-fold increase in 50-nM estradiol-treated cells, $P = 0.002$). However, irrespective of estradiol treatment, luciferase activity was not increased in cells transfected with pERE-luc only.

We next evaluated the luciferase activities of 293T cells cotransfected with the *+102525G>A* construct (Fig. 1). Basal luciferase activities were comparable between the *+102525G* and *+102525A* constructs, and estradiol treatment increased the activity in both constructs in a dose-dependent manner ($P < 0.001$). Although the *+102525G* construct showed a 25.62-fold increase in luciferase activity following 50 nM of estradiol stimulation compared with cells transfected with pERE-luc and pCMV6/ESR1 only, the luciferase activity in *+102525A* transfectant was increased to a greater degree by the same dose of estradiol (fold increase 46.86 ± 2.61; $P = 0.00004$ vs. *+102525G* construct; Fig. 1). This suggests that the *PPARGC1B +102525A* variant is a more potent coactivator than the +102525G variant for ERα.

Comparison of the interaction between PPARGC1B *and ERα according to* +102525G>A

To assess the interaction between *PPARGC1B* and *ERα*, we performed co-IP for FLAG-tagged ERα and HA-tagged PPARGC1B+*102525G* or *A* in 293T cells, as described in the Materials and Methods section. Without 17β estradiol treatment, HA-tagged PPARGC1B was weakly detected by immunoprecipitation using FLAG-tagged ERα, irrespective of the allele. After 17β estradiol treatment, HA-tagged PPARGC1B exhibited greater coprecipitation with ERα, and the interaction level was higher for the *+102525A* than the *+102525G* construct (Fig. 2). The greater ERα-activating ability of the PPARGC1B *+102525A* variant is consistent with its higher ERα-binding affinity than *+102525G*.

Specificity of the PPARGC1B + 102525A *construct in ERα activation*

To evaluate the specificity of the *PPARGC1B* construct as a coactivator for ERα, the luciferase activity in transfected 293T cells was measured after dexamethasone and progesterone treatment (Fig. 3a, b, respectively). Neither dexamethasone nor progesterone increased luciferase activity in the absence of estradiol in both PPARGC1B untransfected and transfected cells, although the PPARGC1B-transfected cells showed a trend toward higher luciferase activity than did cells cotransfected with ESE-luc and ERα only. Stimulation of cells with dexamethasone or progesterone in the presence of estradiol (5 nM) resulted in no additive effect on luciferase activity compared with cells treated with estradiol only. These data suggested that the coactivator activity of PPARGC1B was restricted to ERα and was not involved in the activation of the glucocorticoid or progesterone receptor.

Discussion

In this study, we investigated the functional consequence of the R265Q substitution (caused by the *+102525G>A* polymorphism) of *PPARGC1B*, which was previously reported to be associated with AHR in asthma (Lee *et al.*,

FIG. 1. Luciferase reporter activity induced by ERα and the *PPARGC1B+102525G>A* variants after 17β estradiol treatment. 293T cells were cotransfected with pERE-luciferase construct with or without an ERα-expressing vector, and with allele-specific PPARGC1B constructs for evaluating the effect of PPARGC1B on estradiol-induced ERα activation according to *+102525G* or *+102525A*. Data are presented as fold enhancements of the normalized luciferase activity of each construct to that of pERE-luc only. *$P < 0.05$ compared with the luciferase activity of the transfectant not treated with estradiol. $^{\dagger}P < 0.05$ and $^{\#}P < 0.01$ compared with the luciferase activity of the +102525G transfectant treated with the corresponding dose of estradiol. The data are mean ± SE of six independent experiments. PPARGC1B, peroxisome proliferator-activated receptor gamma coactivator 1 beta.

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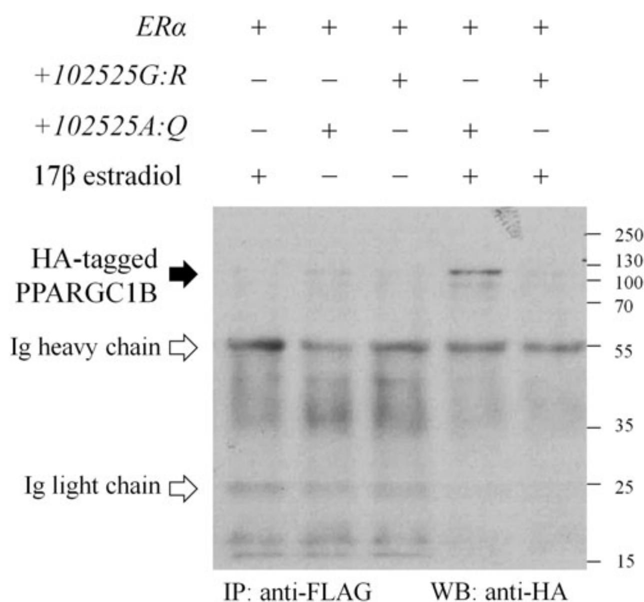

FIG. 2. Coimmunoprecipitation of FLAG-tagged ERα with HA-tagged PPARGC1B according to the PPARGC1B+102525G>A variant. Following cotransfection of 293T cells with both constructs, the cell lysate was precipitated and then detected using anti-FLAG and anti-HA antibodies, as described in the Materials and Methods section. HA, hemagglutinin.

2011). Because *PPARGC1B* acts as a coactivator for ER, we assessed the difference in coactivator activity between +102525G and +102525A using 293T cells cotransfected with pERE-luciferase reporter system, pCMV6/ERα, and pCMV4/PPARGC1B *+102525G>A* constructs, followed by β17 estradiol stimulation. We found that *+102525A* was a superior coactivator of ERα. To the best of our knowledge, this is the first report that the *+102525G>A* SNP (R265Q) of *PPARGC1B* could affect AHR in asthma by modulating the activity of ERα in a genetically different way.

The inflammatory and remodeling process in asthma may be modulated by several transcription factors, such as the GR, STAT-6, and peroxisome proliferator-activated receptor (PPAR) (Rahman and MacNee, 1998; Roth and Black, 2006). Activation of some transcription factors requires the function of coactivators. The human *PPARGC1B* gene is located on chromosome 5q33.1 and comprises 12 exons spanning approximately 117 kb (Andersson *et al.*, 2001). Genome-wide linkage studies identified chromosome 5q31 as a major candidate region linked to asthma and AHR in several ethnic groups (Postma *et al.*, 1995; Xu *et al.*, 2001).

To date, breast cancer and obesity have been reported to be associated with polymorphisms of the *PPARGC1B* gene. Analyses of *BRCA1/2* mutation-negative familial breast cancer patients revealed that a *649G>C* transversion in exon 4 of the *PPARGC1B* gene, resulting in an Ala-to-Pro (A203P) substitution, is associated with increased familial breast cancer risk for heterozygous and homozygous variant allele carriers (Wirtenberger *et al.*, 2006). Interestingly, a significant synergistic interaction exists between the genetic polymorphisms within PPARGC1B and ERα in ER-positive breast cancer (Li *et al.*, 2011). In addition, the common *649G* (corresponding to alanine at position 203) allele is a risk factor for the development of obesity in Caucasians (Andersen *et al.*, 2005).

Considering the biological effect of *PPARGC1B* on *ER*, *PPAR*, and *GR*, which are essential for energy and lipid metabolism (Andersson *et al.*, 2001), an association of the *PPARGC1B* gene with the development of breast cancer and the risk of obesity is biologically plausible. In our previous study, the *PPARGC1B 649G>C* variant was not detected by direct sequencing of DNA from 24 Korean subjects, although the frequency of this variant in the Caucasian population is around 8% (Andersson *et al.*, 2001). Furthermore, the association between *649G>C* and obesity was not replicated in Korean subjects (Park *et al.*, 2006).

PPARGC1B has also been shown to control hepatic gluconeogenesis, an important component of the pathogenesis of both type 1 and type 2 diabetes (Lin *et al.*, 2002). Interestingly, a diabetes-related change in AHR has been reported by human and experimental studies. AHR is increased in patients with type 2 diabetes during the first 3 months of insulin therapy (Terzano *et al.*, 2009). Experimental animal data also demonstrated the effect of insulin on mast cell degranulation (Cavalher-Machado *et al.*, 2004) through enhancement of Th2-mediated responses and activation of NKT cells (Araujo *et al.*, 2004). Furthermore, PPARs reduced antigen-induced airway hyperresponsiveness, lung inflammation, eosinophilia, cytokine production, and GATA-3 expression, as well as serum levels of antigen-specific IgE in a murine model of human asthma (Woerly *et al.*, 2003).

The expression of PPAR is augmented in the bronchial submucosa, the airway epithelium, and the smooth muscle of steroid-untreated asthmatics compared with control subjects (Huang *et al.*, 2005). Our recent studies showed that the *+82466C>T* polymorphism and haplotype 1 of the PPARG gene may be linked to an increased risk of both asthma and aspirin hypersensitivity in asthma (Oh *et al.*, 2009a, 2009b). Based on these data, it is presumed but not demonstrated that dysregulation of the *PPARGC1B* gene may reduce PPAR binding to NR-responsive elements in DNA. This may lead to attenuation of the anti-inflammatory action of PPAR on allergic inflammation, resulting in AHR.

Because *+102525G>A* is located in exon 5, this SNP may affect the production of an alternatively spliced variant lacking exon 4 (which encodes NR1), which is required for interactions with other proteins. We previously reported that both *–427C>T* in the promoter and *+102525G>A* were strongly associated with AHR (Lee *et al.*, 2011). In functional validation, although *–427C>T* affected PPARGC1B promoter activity followed by alternation of the mRNA expression, the levels of both the full-length PPARGC1B mRNA and the alternatively spliced variant did not differ according to the *+102525G>A* genotype. In addition, linkage disequilibrium coefficients between two SNPs were very low in our previous study subjects ($|D'| = 0.49$ and $r^2 = 0.03$) and in Asian population (Han Chinese and Japanese population, $0.44 < |D'| < 0.52$ and $0.01 < r^2 < 0.05$, from HapMap database). These data excluded any effect of the *+102525G>A* on the mRNA expression and the synthesis of splicing variants (Lee *et al.*, 2011).

ERα acts as a critical regulator of AHR by modulating the expression and function of muscarinic receptors (Carey *et al.*, 2007). There are five subtypes of muscarinic receptors

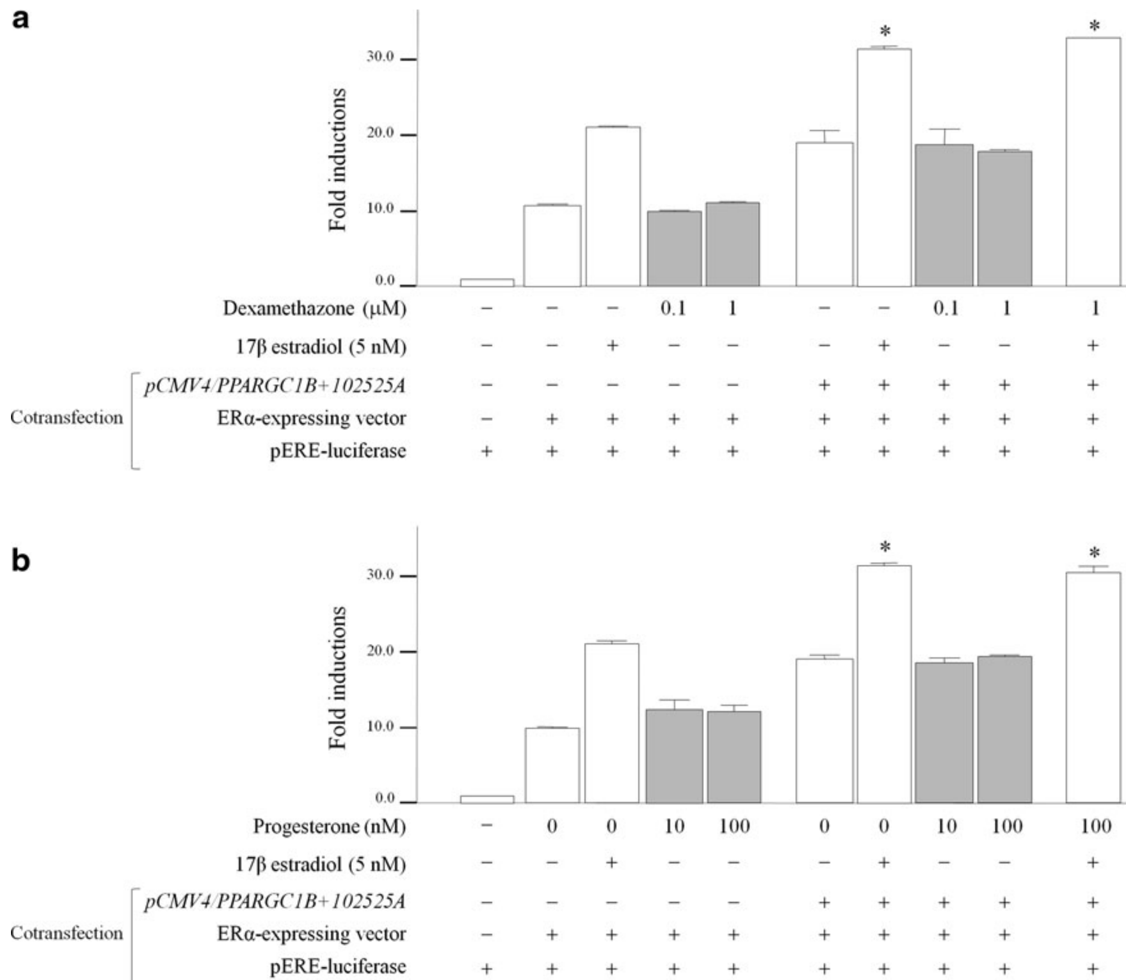

FIG. 3. The specificity of transfected ERα activation by PPARGC1B cotransfection. Luciferase activity was measured after treatment with dexamethasone **(a)** and progesterone **(b)** in 293T cells transfected with pERE-luciferase, an ERα-expressing vector, and *PPARGC1B+102525A*. $^{*}P < 0.05$ compared with the luciferase activity of 293T cells not transfected with a PPARGC1B construct and not treated with estradiol.

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(CHRM1–M5), three of which (CHRM1, CHRM2, and CHRM3) exert physiological effects in the airway (Roffel *et al.*, 1988; Mak and Barnes, 1990). Acetylcholine in the airway stimulates postjunctional CHRM3, which causes smooth muscle constriction, and prejunctional M2 autoreceptors, which result in reduced release of acetylcholine (Gosens *et al.*, 2006). In addition, estrogen induces the expression of β2 adrenoreceptor (ADRB2), which causes bronchodilation (Wheeldon *et al.*, 1994).

Our previous study reported that *PPARCG1B +102525G>A* was not associated with the risk of asthma, but that asthmatics possessing *PPARCG1B +102525A* were less sensitive to methacholine than were subjects with the *+102525G* allele (Lee *et al.*, 2011). The results of this study showed that *+102525A* was a more effective coactivator of PPARCG1B than was *102525G* with regard to the activation of ERα. Thus, our observations are consistent with several lines of evidence drawn from studies using animal models and humans showing that estrogen reduces airway responsiveness (Villa *et al.*, 1990; Pang *et al.*, 2002; Matsubara *et al.*, 2008); our data also indicate that *+102525A* may be involved in the ERα-mediated expression of genes that attenuate AHR—such as *CHRM2* or *ADRB2*—in the airway. This possibility should be evaluated in further studies.

This study had several limitations. First, although PPARGC1B is known to be expressed by vascular smooth muscle cells and cardiomyocytes (Patten and Arany, 2012; Guo *et al.*, 2013), in which it is involved in energy metabolism and regulation of mitochondrial protein expression, no direct evidence is available from a human study on PPARGC1B expression in cells that express ER and play central roles in AHR development, such as airway smooth muscle cells or mast cells (Zhao *et al.*, 2001; Townsend *et al.*, 2012). Because the ERα and PPARGC1B cotransfected 293T cells used in this study could not fully reflect the biological interaction between ERα and PPARGC1B, the physiological features and roles of their interaction in the development of AHR in asthma should be evaluated using human samples. Second, we focused on the interaction between PPARGC1B and ERα and the effects of the alleles on this interaction. In IP assay, we had not directly confirmed that the same amount of ERα was captured and loaded in each lane, because the observed even intensities of immunoglobulin heavy and light chains could represent the

same amount of ERα loaded. This means that a possibility of loading bias could not be excluded. Furthermore, other factors that use PPARGC1B as a coregulator, such as PPAR and GR, have been reported to be involved in the development and modulation of AHR (Goto *et al.*, 2008; Takeda *et al.*, 2009). Thus, the effect of PPARGC1B and its polymorphisms on the regulation of these factors should be evaluated in further studies. Third, ERα and its polymorphisms were reported to be associated with AHR in female asthmatics (Dijkstra *et al.*, 2006; Matsubara *et al.*, 2008). Thus, a possible interaction between ERα and PPARGC1B polymorphisms in their transactivation should be evaluated. Finally, we did not assess the effect of PPARGC1B variants, such as CHRM2 and ADRB2, on the expression of AHR-related receptors through ERα coactivation because co-transfected 293T cells might be inappropriate for this purpose. Thus, to clarify the direct relationship between *PPARGC1B* and AHR, further investigations using primary cells and lung tissues from subjects with AHR should be performed.

Conclusion

In summary, we report that the *+102525G>A* SNP in exon 5 of the *PPARGC1B* gene may affect AHR development through ER activation. These data on the genetic polymorphisms of the *PPARGC1B* gene may facilitate development of novel methods for identifying genetic markers of AHR.

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Disclosure Statement

No competing financial interests exist.

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