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Estrogen receptor beta signaling inhibits PDGF induced human airway smooth muscle proliferation

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ABSTRACT

Airway smooth muscle (ASM) cell hyperplasia driven by persistent inflammation is a hallmark feature of remodeling in asthma. Sex steroid signaling in the lungs is of considerable interest, given epidemiological data showing more asthma in pre-menopausal women and aging men. Our previous studies demonstrated that estrogen receptor (ER) expression increases in asthmatic human ASM; however, very limited data are available regarding differential roles of ERα vs. ERβ isoforms in human ASM cell proliferation. In this study, we evaluated the effect of selective ERα and ERβ modulators on platelet-derived growth factor (PDGF)-stimulated ASM proliferation and the mechanisms involved. Asthmatic and non-asthmatic primary human ASM cells were treated with PDGF, 17β-estradiol, ERα-agonist and/or ERβ-agonist and/or G-protein-coupled estrogen receptor 30 (GPR30/GPER) agonist and proliferation was measured using MTT and CyQuant assays followed by cell cycle analysis. Transfection of small interfering RNA (siRNA) ERα and ERβ significantly altered the human ASM proliferation. The specificity of siRNA transfection was confirmed by Western blot analysis. Gene and protein expression of cell cycle-related antigens (PCNA and Ki67) and C/EBP were measured by RT-PCR and Western analysis, along with cell signaling proteins. PDGF significantly increased ASM proliferation in non-asthmatic and asthmatic cells. Treatment with PPT showed no significant effect on PDGF-induced proliferation, whereas WAY interestingly suppressed proliferation via inhibition of ERK1/2, Akt, and p38 signaling. PDGF-induced gene expression of PCNA, Ki67 and C/EBP in human ASM was significantly lower in cells pre-treated with WAY. Furthermore, WAY also inhibited PDGF-activated PCNA, C/EBP, cyclin-D1, and cyclin-E. Overall, we demonstrate ER isoform-specific signaling in the context of ASM proliferation. Activation of ERβ can diminish remodeling in human ASM by inhibiting pro-proliferative signaling pathways, and may point to a novel perception for blunting airway remodeling.

1. Introduction

Asthma is characterized by a chronic inflammation of conducting airways in association with airway hyperresponsiveness (AHR) and remodeling (Prakash, 2013, 2016; Prakash and Martin, 2014; Lazaar and Panettieri, 2005; Sathish et al., 2011; Dekkers et al., 2009). Structural changes in the airway walls, induced by a vicious circle of injury and repair processes collectively represent airway remodeling. Increased airway smooth muscle (ASM) mass is a hallmark of airway remodeling which causes airway narrowing and obstruction (Wang et al., 2016; Rydell-Törmänen et al., 2012). Altered extracellular matrix also contributes to airway remodeling in asthma (Gerthoffer, 2008; Hershenson et al., 2008; Koziol-White et al., 2011; Roscioni et al., 2010; Coraux et al., 2008; Bossé et al., 2008). Studies including our own supports the concept that ASM proliferation augments ECM generation and deposition in asthmatic airways (Dekkers et al., 2009; Rydell-Törmänen et al., 2012; Coraux et al., 2008; Bossé et al., 2008; Freeman et al., 2017; Royce et al., 2012; Lagente and Boichot, 2010). Multiple studies suggest that ASM cell migration towards the airway epithelium in response to inflammatory mediators, such as platelet-derived growth factor (PDGF) contributes to airway remodeling (Gerthoffer, 2008; Suganuma et al., 2012; Carlin et al., 2003; Hirst et al., 1992; Ingram and Bonner, 2006). As a result, the ASM layer in asthmatic patients is in close proximity to airway epithelial cells (Joubert and Hamid, 2005; James et al., 2002), which may lead to increased airway hyper-responsiveness. Thus better understanding of mechanisms that contribute

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to enhanced ASM proliferation are important in the context of developing novel strategies to address asthma.

An emerging aspect of asthma is the clinical recognition that prevalence of asthma is greater in boys than in girls during prepubescent ages (Sathish et al., 2015; Bonds and Midoro-Horiuti, 2013; Bjornson and Mitchell, 2000; Caracta, 2003; Carey et al., 2007) with this trend reversing after puberty to become more prevalent in adult women (Sathish et al., 2015a,b; De Marco et al., 2000; Melgert et al., 2007). In adult women, changes in symptom severity and number of exacerbations correlated to changes in hormonal status (phases of the menstrual cycle, pregnancy, and menopause) (Bjornson and Mitchell, 2000; Caracta, 2003; Carey et al., 2007; Townsend et al., 2012). Thus, the potential role of sex steroids in the modulation of asthma pathophysiology becomes relevant in the context of understanding sex differences in this disease. Here, estrogens may be particularly important given clinical data showing that modulating estrogen levels correlate to changes in asthma symptoms.

The effect of estrogen is mediated by two receptors (estrogen receptors, ERα and β), which are known to act as ligand-dependent transcription factors (Laudet and Gronemeyer, 2002). However, several studies including our own have shown that ERs are also capable of acting in a non-genomic fashion, for example modulating calcium responses in ASM cells (Townsend et al., 2010, 2012; Townsend et al., 2012a,b), and furthermore acting genomically to modulate signaling pathways relevant to airway disease (Sathish et al., 2015a,b; Prossnitz et al., 2008; Marino et al., 2006).

Previously we showed that in human ASM, physiologically-relevant concentrations of estrogens act via ERs and the cAMP pathway to non-genomically reduce $[Ca^{2+}]_i$, thus promoting bronchodilation (Sathish et al., 2015a,b; Townsend et al., 2010, 2012). Our recent work showed the differential expression of ERα and ERβ in asthmatic and non-asthmatic ASM, and found ERβ expression is significantly greater in asthmatic ASM in both males and females (Aravamudan et al., 2017), but the downstream signaling has not been elucidated. There have been studies demonstrating that 17β-estradiol increases ASM number (Stamatiou et al., 2011) and vascular smooth muscle proliferation (Cheng et al., 2009; Muka et al., 2016) while other studies show ERα and ERβ agonists causing significant inhibition of proliferation of vascular smooth muscle cells (Muka et al., 2016; Li et al., 2016, 2017). There are currently no data on ER effects on human ASM proliferation, especially in the context of inflammation or asthma. The present study hypothesizes that differential activation of ERs plays a role in ASM proliferation during inflammation. We investigated the role of estrogen along with ERα and ERβ agonists in human ASM proliferation in the presence of PDGF as mitogenic stimulus, with the idea that if ERβ is more abundant in asthmatic ASM, this receptor isoform has greater contribution towards ASM proliferation.

2. Materials and methods

2.1. Chemicals, drugs/inhibitors, antibodies

Cell culture reagents and other cell culture supplies including fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium F/12 (DMEM/F12) were purchased from Invitrogen (Carlsbad, CA). Pharmacological agonists for ER and its isoforms such as 17β-estradiol (E_2), ERα-agonist (PPT, Propyl pyrazole triol; THC, Tetra Hydro Chrysenediol), G-protein-coupled estrogen receptor 30 (GPR30/GPER) agonist (G1), and ERβ-agonist (WAY-200070; FERB-033; DPN, Diaryl-Propio-Nitrile) were obtained from Tocris (Minneapolis, MN). Human recombinant PDGF-BB was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-13 (IL-13) were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). Chemicals and supplies were from Sigma (St. Louis, MO) unless otherwise specified. ESR1, ESR2 and negative siRNA obtained from Dharmacon (Lafayette, CO, USA) and Ambion (Austin, TX, USA). Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology, Inc (Dallas, TX) unless otherwise mentioned. Estrogen Receptor α Monoclonal Antibody (33, Catalog # MA1-310) and Estrogen Receptor β Monoclonal Antibody (PPZ0506, Catalog # MA5-24807) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Akt1 (Cat# 9272S), pAkt1 (Cat# 4060S), p38 (Cat# 8690S), p-p38 (Cat# 4511S), antibodies were obtained from Cell Signaling, β-actin antibody from Sigma-Aldrich (Cat# A5316), and the remainder (ERK1/2 (Cat# sc-94), pERK1/2 (Cat# sc-7383), PCNA (Cat# sc-25280), C/EBP (Cat# sc-365318) Cyclin-D1 (Cat# sc-8396), and Cyclin-E (Cat# sc-481)) from Santa Cruz. The secondary antibodies m-IgGκ-HRP (Cat# sc-516102), Goat anti-rabbit IgG-HRP (Cat# sc-2004) were obtained from Santa Cruz and ECL anti-mouse IgG-HRP (Cat# NA931V) from GE healthcare UK Ltd.

2.2. Human ASM cells

The technique for isolating human ASM cells has been previously described (Abcejo et al., 2012; Vohra et al., 2013; Sathish et al., 2015a,b). Briefly, third to sixth generation human bronchi were obtained from lung specimens incidental to patient thoracic surgery at Mayo Clinic for focal, non-infectious causes (typically lobectomies for focal cancers). Normal lung areas were identified with the help of a pathologist (protocol approved by Mayo Clinic Institutional Review Board). We used samples from both male and female adults of ages from 21 to 60 yr. Samples were denuded of epithelium and ASM tissue enzymatically dissociated to generate cells that were used for experiments. For cells, cultures (< 3rd passage) were maintained under standard conditions of 37 °C (5% CO_2, 95% air). Cells were serum starved for 24 h prior to experimentation. Experiments were conducted in Hanks' Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA).

2.3. Cell treatment

Fully confluent T-75 flasks of ASM cells were trypsinized mixed in 10% FBS (Charcoal Stripped) Growth medium (DMEM/F12/1% AbAm), counted and seeded ~10,000 cells/well into 96-well culture plates. Cells were allowed to adhere overnight, washed twice with phosphate buffered saline (1 × PBS) and incubated in serum free medium (without FBS) for 24 h to mature and synchronize the growth of cells. ASM cells were treated with 17β-estradiol (E_2, 1 nM), ERα-agonist (PPT, 10 nM), or ERβ-agonist (WAY, 10 nM), GPR30/GPER agonist (G1, 1 nM) with 1% FBS in the media. After 2 h of pre-incubation with respective treatment groups, cells were then exposed to PDGF (2 ng/ml) for 24 h. DMEM/F12 with 1% FBS alone served as a vehicle. For a set of experiments, 20 ng/ml TNFα, 50 ng/ml IL-13, 1 nM G1 and 10 nM each of THC, FERB-033 and DPN were used.

2.4. Small interfering RNA transfection

Technique to knock down the Estrogen receptor α and β by interfering RNA (siRNA) has also been previously described (Aravamudan et al., 2012). Briefly, human ASM cells were grown on 96 well plate or 100 mm plates to approximately 50% confluence. Transfection was achieved using 20 nM siRNA and Lipofectamine 3000 transfection reagent (Invitrogen) in DMEM F-12 lacking FBS and antibiotics. Fresh growth medium was added after 6 h and cells analyzed after 48 h. ESR1 silencer select Pre-designed siRNA (Ambion, Austin, TX, USA, Catalog# 4392420), ON-TARGETplus Human ESR2 (2100) siRNA – Individual (Dharmacon, Lafayette, CO, USA, Catalog # J-003402-13-0005) were used to knock down Estrogen receptor α and β respectively. As a negative control, the Silencer Negative Control #1 (Cat# AM4611) from Ambion was used. Knockdown efficacy and specificity was verified by Western blot analysis. The effect of siRNA on proliferation in presence and absence of PDGF, WAY or PPT were analyzed by MTT assay

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