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Role of G protein-coupled estrogen receptor 1 in modulating transforming growth factor-β stimulated mesangial cell extracellular matrix synthesis and migration

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ABSTRACT

Estrogen has been demonstrated to exert beneficial effects on kidney; however, the role of G protein-coupled estrogen receptor 1 (GPER) is still uncertain. In the present study, we investigated the effect of 17β-estradiol and GPER agonist Fulvestrant on extracellular matrix production under transforming growth factor-β1 (TGF-β1) stimulation in human and rat mesangial cells. As a result, 17β-estradiol and Fulvestrant inhibit TGF-β1-induced type IV collagen and fibronectin expression in a dose-dependent manner, by suppressing acute Smad2/3 phosphorylation and Smad4 complex formation. Furthermore, estrogen and Fulvestrant also down-regulate Smad signaling by promoting ubiquitin/proteasome-dependent Smad2 degradation. These effects could be abrogated by receptor antagonist G-15 or GPER gene knockdown. GPER is also required for estrogen and Fulvestrant to regulate mesangial cell migration in response to TGF-β1. To conclude, GPER is crucial in modulating glomerular mesangial cell function including extracellular matrix production and migration.

1. Introduction

Gender has a crucial impact on progress and prognosis of chronic kidney diseases (Eriksen and Ingebretsen, 2006; Neugarten et al., 2000b) and estrogen slows down its progression (Silbiger and Neugarten, 2008). Glomerular mesangial cells, of which hypertrophy persists in the progression of diabetic nephropathy and are intimately involved in renal fibrosis, produce and secrete extracellular matrix which mainly consists of type IV collagen and fibronectin, maintaining glomerular capillary structure (Abboud, 2012; Eisel et al., 2013). Mesangial cell migration alterations are also involved in multiple renal diseases, and are crucial part of mesangial cell function loss during pathological conditions (Crean et al., 2002; Martin et al., 2002). 17β-Estradiol and selective estrogen receptor modulators (SERMs) suppress excessive mesangial matrix synthesis (Kwan et al., 1996; Neugarten et al., 2000a; Silbiger et al., 1998, 1999) and modulate signals induced by mechanical strain (Krepinsky et al., 2002) in cultured mesangial cells. Meanwhile, 17β-estradiol attenuates mesangial expansion during diabetic nephropathy in rats, by regulating extracellular matrix metabolism, as well as transforming growth factor-β expression and signaling (Dixon and Maric, 2007; Mankhey et al., 2005).

There are several major pathways involved in chronic kidney diseases characterized by mesangial dysfunction and other pathological alterations. Transforming growth factor-β (TGF-β) synthesis is enhanced during renal diseases including mesangial injuries, and TGF-β mediates high glucose- and mechanical strain-induced mesangial matrix synthesis (Riser et al., 1998; Wada et al., 2002). TGF-β is the key mediator of renal injuries, and its binding to TGF-β receptors leads to R-Smads (Smad2 and Smad3) phosphorylation. Smads protein complex (Smad2/3/4), of which formation ensures Smad signal transduction, translocates into the nucleus and regulates target genes (e.g. genes encoding extracellular matrix including type IV collagen, fibronectin, etc.) transcription (Abdollah et al., 1997). TGF-β also up-regulates connective tissue growth factor (CTGF) gene expression through Smad pathways (Chen et al., 2002). Excessive CTGF, together with TGF-β, induces abnormal human glomerular mesangial cell migration *in vitro*

Abbreviations: GPER, G protein-coupled estrogen receptor 1; TGF-β, transforming growth factor-β; SERM, selective estrogen receptor modulators; CTGF, connective tissue growth factor; ER, estrogen receptor.

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studies (Weston et al., 2003). Therefore, TGF-β stimulation mimics the pathologocial processes in mesangial cells under various damaging stimulations, and an observation of the cell responses upon drug treatment may provide clues to the treatments which aim to attenuate or prevent mesangial cell injuries during acute or chronic glomerular diseases.

Estrogen interacts with classical estrogen receptors (ER) and then regulates downstream target gene transcription. Estrogen crosstalks with TGF-β signaling through Smad2/3 (Band and Laiho, 2011; Matsuda et al., 2001). And it is demonstrated that estrogen inhibits TGF-β/Smad signaling by promoting R-Smad degradation (Ito et al., 2010). Meanwhile, 17β-estradiol, SERMs and Fulvestrant, also have been proved to agonize the non-classical G protein-coupled estrogen receptor 1 (GPER, GPR30) (Fliardo et al., 2002; Thomas et al., 2005). GPER is a 17β-estradiol-binding receptor, which locates on cell membrane and expresses in various tissues including central nervous system, cardiovascular system, endocrine system, immune system, reproductive system, musculoskeletal system and renal system (Prossnitz and Barton, 2011, 2014). Previous research has primarily proved a role of GPER in regulating TGF-β/Smad signaling. In breast cancer cells, 17β-estradiol inhibits TGF-β signaling and function via ERK activation through GPER (Kleuser et al., 2008). GPER also regulates MAPK and signaling of other growth factors (Fliardo et al., 2002). GPER agonists also exert renoprotective effect on diabetic and hypertensive nephropathy (Li et al., 2013; Prossnitz and Barton, 2011). In addition, in a recent work, we discovered that Fulvestrant, which we intended to block ER, exerted a surprising additive effect to a GPER-dependent xenoestrogen in regulating excessive mesangial extracellular matrix accumulation induced by high-glucose condition as well as direct TGF-β stimulation (Li et al., 2013). For little is known about the function of GPER in glomerular mesangial cells, we hypothesize that GPER affects TGF-β/Smad signaling and restores mesangial cell function which is undermined by detrimental stimulations.

2. Material and methods

2.1. Cell culture and treatments

Human mesangial cell line (Li et al., 2013; Sraer et al., 1996) was a kind gift from Prof. Bicheng Liu (Institute of Nephrology, Southeast University School of Medicine, Nanjing, China). Rat mesangial cells were, as described previously (Li et al., 2013), separated from 4-week-old female Sprague–Dawley rats by mincing the kidney cortex and sieving, and passage 3–10 were used. Human and rat mesangial cells were cultured in RPMI 1640 medium (HyClone Laboratories, Inc., South Logan, UT, USA) supplemented with 10% (v/v, human) or 20% (v/v, rat) fetal bovine serum (Hyclone), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone) maintained at 37 °C in a humidified atmosphere containing 5% CO_2. At 70% confluence, mesangial cells were starved in serum-free medium for 24–48 h and then exposed to 10^{-11} M to 10^{-7} M of 17β-estradiol ((17β)-Estra-1,3,5(10)-triene-3,17-diol, Tocris Biosciences, Bristol, UK), Fulvestrant (ICI 182,780, 7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfin-yl]nonyl]estra-1,3,5(10)-triene-3,17-diol, Sigma–Aldrich, St. Louis, MO, USA), 10^{-6} M of G-15 ((3aS*, 4R*, 9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline, Tocris Biosciences) (Dennis et al., 2009) or simultaneously stimulated for varying period by 2 ng/ml Recombinant transforming growth factor-β1 (TGF-β1, R&D Systems, Minneapolis, MN, USA). TGF-β1 was reconstituted and diluted in 4 mM hydrochloric acid containing 1 mg/ml bovine serum albumin, and other chemicals were dissolved in dimethyl sulfoxide. Control groups supplied with vehicles were included in all experiments. All experiments were conducted at least three separate times in triplicate. The study was reviewed and approved by the Ethics Committee of China Pharmaceutical University.

2.2. siRNA transfection

Cells were grown to sub-confluency and transfected with GPER siRNA or negative control siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using PepMute™ siRNA Transfection Reagent (SignaGen Laboratories, Ijamsville, MD, USA). The mRNA level of GPER at different time points (24 h, 48 h, and 72 h) after three concentrations (5 nM, 10 nM, 15 nM) of siRNA transfection was determined, and the knockdown efficiency at the optimized concentration was below 1%. After 48 h of transfection, cells were exposed to drugs and were harvested at indicated times for different experiments.

2.3. Cell lysate preparation

Cell lysates for immuneprecipitation and Western blot were prepared by washing cells twice with ice-cold phosphate-buffered saline followed by cell lysis in RIPA buffer (Byotime, Nantong, China) supplemented with 1% (v/v) Protease Inhibitor Cocktail (Sigma–Aldrich) and 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. Protein concentrations of samples for Western blot were measured using BCA assay kit (Byotime) and mixed with 5× loading buffer containing 0.25 M Tris–HCl (pH 6.8), 0.5 M dithiothreitol, 10% sodium dodecyl sulfate, 0.5% bromophenol blue and 50% glycerol and boiled for 10 min before electrophoresis on 10% acrylamide gels. 10 μg of protein was loaded per lane.

2.4. Immuneprecipitation

Cells were lysed and the extracted proteins were diluted to same concentrations with phosphate-buffered saline. Ubiquitin antibody (Abcam, New Territories, HK) or Smad2/3 antibody (Cell Signaling Technology, Danvers, MA, USA) was added to the protein samples for 2 h at 4 °C to immunoprecipite target proteins, and the immunecomplexes were captured by Protein A/G-Agarose (Abmart, Shanghai, China). The precipitations were collected and dissolved in 1× loading buffer and boiled for 10 min before electrophoresis.

2.5. Western blot

After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the proteins were transblotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Richmond, CA), and membranes were blocked by 5% bovine serum albumin in Tris-buffered saline supplemented with 0.1% Tween-20 for 2 h at room temperature. Membranes were then incubated with primary antibodies against Phospho-Smad2 (Ser465/467) (Cell Signaling Technology), Phospho-Smad3 (Ser423/425) (Cell Signaling Technology), Smad2/3, Smad4 (Cell Signaling Technology), Smad7 (R&D Systems) or β-actin (CMC Scientific, Shanghai, China) at 4 °C overnight. Blots were washed before incubation with the goat anti-rabbit (Sunshinebio, Nanjing, China) or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Abmart) for 2 h at room temperature. The protein bands were visualized by incubating with enhanced chemiluminescence reagent (Super-Signal West Pico, Thermo Scientific, Rockford, IL, USA) before exposure using Bio-Rad ChemiDoc XRS + Imaging System. Protein bands were quantified using ImageJ Software version 1.46r (National Institute of Health, Bethesda, MD, USA). The Western blot results were representative of experiments performed at least three times.