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Research Article

The effects of diosgenin in the Regulation of renal proximal tubular fibrosis

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ABSTRACT

Fibrosis is the important pathway for end-stage renal failure. Glucose has been demonstrated to be the most important fibrogenesis-inducing agent according to previous studies. Despite diosgenin has been demonstrated to be anti-inflammatory, the possible role in fibrosis regulation of diosgenin remain to be investigated. In this study, renal proximal tubular epithelial cells (designated as HK-2) were treated with high concentration of glucose (HG, 27.5 mM) to determine whether diosgenin (0.1, 1 and 10 μM) has the effects to regulate renal cellular fibrosis. We found that 10 μM of diosgenin exert optimal inhibitory effects on high glucose-induced fibronectin expression in HK-2 cells. In addition, diosgenin markedly inhibited HG-induced increase in α-smooth muscle actin (α-SMA) and HG-induced decrease in E-cadherin. In addition, diosgenin antagonizes high glucose-induced epithelial-to-mesenchymal transition (EMT) signals partly by enhancing the catabolism of Snail in renal cells. Collectively, these data suggest that diosgenin has the potential to inhibit high glucose-induced renal tubular fibrosis possibly through EMT pathway.

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Introduction

Diabetic nephropathy is a chronic renal disease that develops as a result of diabetes mellitus (DM), affects approximately 10% of global world population [1]. Numerous studies have demonstrated that glucose is a key factor in experimental models of diabetic kidney disease as well as in patients with diabetic nephropathy [2–5]. Diabetic nephropathy is a common end stage in either type I or type II diabetic patients. The pathogenesis of diabetic nephropathy is thought to involve both metabolic and vascular factors, resulting in kidney fibrosis [6]. Renal fibrosis arises from long-standing inflammation and tissue injury, mainly

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referring to chronic and progressive kidney failure. In renal fibrosis, one report suggested that proximal tubule epithelial cells contributed to 36% of the total fibroblasts pool through EMT [7].

Epithelial-to-mesenchymal transition (EMT) is increasingly being considered as a possible mechanism leading to renal fibrogenesis [8]. In a previous study, the crucial cellular changes have been identified, including cytoskeletal remodeling by a polarized phenotype and changes in transcriptional regulation to increase cell mobility and invasion. E-cadherin and α-smooth muscle actin are the most important proteins involved in EMT [10]. In the process of EMT, a significant decrease in E-cadherin and the increase in α-smooth muscle actin (α-SMA) expression have been demonstrated for years concomitantly with the increase in Snail protein [11]. Snail is a zinc finger transcriptional factor, functions as a regulator to suppress the expression of adhesion molecules from cell death during EMT [12]. The most common biochemical change associated with EMT is the loss of E-cadherin expression. According to Medici et al., repression of E-cadherin was by Snail [13].

Diosgenin is the primary furostanol saponin found in several plants, including *Dioscorea* species (yams) [14]. Diosgenin is a precursor of steroid hormones such as progesterone and anti-inflammatory steroids [15]. In previous studies, diosgenin, a phytoecdysteroid, attenuated renal injury in diabetes models and has been shown to be useful for the maintenance of healthy blood cholesterol levels and anti-inflammatory, [16]. However, literature survey showed no scientific evidence supporting the therapeutic effects of diosgenin in renal fibrosis. Hence, the present study was prompted by the urgent requirement for natural, safe and effective anti-fibrosis agents.

In this study, we use the in vitro model to elucidate the effects of diosgenin in renal fibrosis. This study finds that diosgenin plays a pivotal role on the regulation of renal fibrosis. In addition, diosgenin has been shown to inhibit high glucose-induced renal tubular fibrosis possibly through EMT pathway.

Materials and methods

Cell culture

Human proximal tubule epithelial cells, HK-2 (CRL-2190; American Type Culture Collection, Manassas, VA) is a proximal tubular cell line derived from a normal kidney, was seeded 5×10^5 and cultured in 25 T flasks at a 5% CO_2 and 95% air environment incubator with Nutrient Mixture F-12 [HAM] (Sigma, St. Louis, MO, USA) supplemented included 10% (vol/vol) fetal bovine serum (GIBCO, Grand Island, NY), 2% (v/v) penicillin/streptomycin (Hyclone Labs, Logan, UT), 1% (v/v) L-glutamine, at 37 °C in 95% air–5% CO_2. The cells were trypsinized using 0.05% trypsin-EDTA (GIBCO). The cells were trypsinized by 0.025% trypsin-EDTA (Hyclone Labs, Logan, UT). Prior to exposure to diosgenin, cells were cultured in minimum starvation media (0.1% FBS) for 24 h. For glucose treatments, cells were cultured in culture flasks (Nunclon, Denmark) and treated with vehicle (PBS) or 27.5 mM D-glucose (GIBCO, Grand Island, NY) for 48 h to induce cellular fibrogenesis and different concentrations of diosgenin (Sigma-Aldrich, St Louis, MO) in last 24 h. Three wells were allocated for each treatment, including the negative control (untreated cells).

Cell viability assay

Cells that had been suspended in the culture medium were collected by centrifugation (1200 rpm). The collected fraction (dead cells) was pooled with that collected from originally attached cells, which were trypsinized and collected by centrifugation at 2000 rpm for 10 min. Trypan blue (0.4%) was mixed with an equal volume of the cells mixture; then, the cells were counted using a haematocytometer. Cells were lyzed using lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na3VO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM dithiothreitol and 50 mM PMSF) and use Brilliant Blue G-250 to assay protein content.

Scattering assay

HK-2 cells were seeded in each well of a 24-well plate and incubated overnight in a 37 °C incubator with 5% CO_2. Glucose was added to each well at the final concentration of 27.5 mM for 24 h and pretreated with diosgenin for 24 h at the indicated concentrations. Representative photographs were taken at 200 × magnification using an inverted microscope.

Wound healing assay

To generate confluent cell monolayers, 1 × 105 HK-2 cells per well of a wound healing assay kit (Ibidi, Germany) were cultured 24 h in serum-containing Nutrient Mixture F-12 [HAM] in a 37 °C incubator with 5% CO_2. After 24 h HK-2 cells were incubated with 27.5 mM for 24 h, removed with wound healing assay kit and added with diosgenin (10 μM) in the last 24 h. Cell motility was determined by measuring the percentage of cells. Data are plotted as means with SD. The distance between groups was considered to be statistically significant when ($p<0.05$).

Enzyme-linked immunosorbent assay

We use ELISA kit (Assaypro, St. Charles, MO) to quantify fibronectin in the supernatant of the cultured HK-2 cells, conditioned culture medium was collected and centrifuged at 2000 rpm for 5 min to remove particulates; the clear supernatant was collected and concentrated. The concentrated supernatant was stored at −45 °C until use. The protocols were performed according to the manufacturer's instructions. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. After the absorbance for fibronectin was assayed, the concentration of each protein was determined by interpolating against a standard curve.

Immunoflourescence staining

HK-2 cells were fixed with 4% paraformaldehyde for 15 min, and permeabilzed with cold PBS/0.25% Triton-X-100 for 10 min. There after non specific binding is blocked by blocking 5% BSA in PBS for 40 min. E-cadherin (ab53033; Abcam, Cambridge, UK), α-smooth muscle actin (ab5694; Abcam, Cambridge, UK), fibronectin (ab23751; Abcam, Cambridge, UK) and Snail (ab85931; Abcam, Cambridge, UK) were stained by sequentially incubating with rabbit antihuman fibronectin antibody for 1 h and FITC-conjugated to goat anti-rabbit immunoglobins (Molecular Probes) for 1 h with PBS wash after each incubation. Finally, nuclei were labeled with mounting medium including DAPI (DAPI, Santa Cruz

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