Original Research Article

Diosgenin inhibits cell proliferation of primary human thyrocytes via downregulation of PI3K/Akt signaling pathway

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Abstract

Purpose: To determine the potential influence of diosgenin on proliferation of human thyrocytes and its possible mechanism.
Methods: Primary human thyrocytes were cultured and treated with diosgenin at various time intervals. Anti-proliferative activity was determined by MTT assay. Cell proliferation was evaluated by EdU assay while cell cycle was analyzed using fluorescence-activated cell sorting (FACS) method. Protein expression of p21 (CIP1), p27 (KIP1), cyclins, protein kinase B (Akt), phosphatidylinositol 3 kinase (PI3K) and p-Akt was determined by the western blot.
Results: Diosgenin inhibited proliferation of primary human thyrocytes and caused G0/G1 arrest in a concentration-dependent manner. It also downregulated cyclin D1 and phosphorylation of PI3K and Akt, but upregulated p21 and p27.
Conclusion: Inhibition of proliferation of primary human thyrocytes by diosgenin occurs via downregulation of PI3K/Akt signaling pathway. Therefore, diosgenin can be developed as a potential drug for the treatment of thyroid disease.

Keywords: Diosgenin, Proliferation, Primary human thyrocytes, PI3K/Akt signaling pathway

INTRODUCTION

Graves' disease (GD) is a common autoimmune thyroid disease. Diffusion of thyroid goiter is a characteristic feature of GD in which cell proliferation is important in the pathogenesis, as the size of thyroid is proportional to the length of time of GD medical treatment [1]. It is hence important to explore the mechanism for thyroid goiter formation to find effective therapies for GD.

Instead of using radioactive iodine and thyroid surgery, antithyroid drugs, including carbimazole, methimazole, and propylthiouracil are the mainstream therapies for uncomplicated GD [2]. There are, however, drawbacks of these treatments including high recurrence, long treatment course and low remission rate for large goiters. Antithyroid drugs inhibit synthesis of thyroid hormone in large goiter; however, they

are sometimes non-effective in remitting goiter and often cause mental stress on patients [3]. Anti-proliferative approaches might be a better way to treat GD, especially for large goiters, but such approaches have not been studied yet.

Diosgenin, originally extracted from wild yam roots (*Dioscorea villosa*) [4], is a steroidal saponin also found in plants such as *Dioscorea* species, fenugreek and *Costus speciosus* [5]. Diosgenin is traditionally used for diabetes treatment [6], skin aging [7], hyper-cholesterolemia [8], and gastrointestinal ailments [9]. It exhibits various biological activities, including anticancer activity, cardiovascular protection, anti-diabetic activity, neuroprotection, immunomodulation, and skin protection [10]. The inhibitory effect of diosgenin on thyrocyte proliferation has also been previously described [11]; however, it is not clear if diosgenin exerts any effects on human thyroid follicles.

This work aimed to evaluate the role of diosgenin on proliferation of primary human thyroid cells and to explore the mechanism of its action.

EXPERIMENTAL

Thyroid cell culture

Human thyrocytes were from euthyroid patients who had benign follicular nodules surgery. Dissected and minced tissues were used in the experiments. Reagents used were purchased from Sigma-Aldrich (San Francisco, USA) unless otherwise stated. The resulting samples were centrifuged at 500 g in Ca^{2+}/Mg^{2+}-free Hanks' salt solution, in which the samples were then shaken with type I collagenase (200 units/mL), trypsin (0.25 %) and heat-inactivated dialyzed chicken serum (GIBCO, 0.75 mg/mL) at 37 °C for 60 min. Supernatant was washed with Dulbecco's Modified Eagle Medium (DMEM) and F12 culture medium and then centrifuged at 500 g [12].

The resulting tissues were added to a mixture of DMEM and F12 culture media supplemented with new-born bovine serum (NBS, 10 %), thyroid stimulating hormone (TSH, 2 m units/mL), penicillin (100 units/mL), streptomycin (100 units/mL) and amphotericin B (2 µg/mL). Cells were maintained in DMEM/F12 media for primary culture and were kept at 37 °C in a humidified atmosphere with 5 % CO_2. Supernatant was removed 24 h subsequent to incubation. Within 5 days, the cells were observed to reach 70 – 80 % confluence. Consent was obtained from all and the experimental method was approved by Yancheng First People's Hospital Ethics Committee (ethics ref no. 2014011) and also followed both the guidelines of WHO International Code of Ethics for Human Biomedical Research and Helsinki Declaration [13,14].

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay

Anti-proliferative activity of diosgenin was measured using MTT assay. Cells were seeded in 96-well microtiter plates (8000 cells/well) and were incubated for 24 h, after which the growth medium was replaced with DMEM/F12 (supplemented with NBS (0.2 %), penicillin, streptomycin and amphotericin B) in which the cells were starved at 37 °C overnight. Diosgenin was added to the cells at 24, 48 and 72 h, respectively. MTT solution (10 µL, 12 mM, Beyotime, China) was added to each well with cells and the same amount of MTT solution was added to the pure medium as the negative control. Posterior to the complete formation of formazan crystal dimethyl sulfoxide (DMSO, 10 µL) was added. Absorbance was determined in a spectrophotometer at a wavelength of 570 nm.

5-Ethynyl-2'-deoxyuridine (EdU) assay

The influence of diosgenin on proliferation of primary human thyrocyte cells were assessed using the EdU assay kit (Ribobio, China). Thyrocytes were maintained in 96-well plates. To the cells diosgenin was added subsequent to overnight starvation and were left incubating for 24 or 48 h. EdU (50 µM/L) was added to the cells and incubated at 37 °C for 4 h, after which formaldehyde (4 %) was added to fix the cells, which were left at room temperature (20 - 25 °C) for 30 min. The following Triton X-100 (0.5 %) treatment lasted for 20 min for permeabilization. The permeabilized cells were then treated with Apollo® reaction cocktail (100 µL/well) and incubated for 30 min subsequent to washing the cells with PBS. DNA-staining with bisbenzimide (100 µL, 5 µg/mL) was carried out and then left for 30 min and examined under microscope.

Flow cytometry: fluorescence-activated cell sorting (FACS)

Cell cycle was analyzed by FACS. Diosgenin was added after overnight starvation to the cells, which were harvested and stained with propidium iodide (Beckman Coulter, USA). The resulting cells were examined on EPICS Elite ESP (Beckman Coulter, USA). Ratios of cells in different growth phases, G_0/G_1, S, and G_2/M, were calculated and processed using the Coulter's Cytologic.

SDS-PAGE and western blot

Pre-washed (with PBS) cells were lysed in RIPA buffer at 0 °C for 20 min. The resulting protein lysates were centrifuged at a speed of 12,000 rpm at 4 °C for 10 min, after which the supernatants at upper layer were either used immediately or preserved at -80 °C. Concentrations of protein were determined using the bicinchoninic acid assay (Pierce, USA). 40 µg of protein was heated at 60 °C for 30 min for each sample and separated by SDS-PAGE.

The separated protein was transferred onto nitrocellulose membranes, which were then blocked with 5 % skimmed milk for 1 h and then kept at 4 °C overnight with primary antibodies (cyclin D1 (1: 400), cyclin B1 (1: 1000), PI3K (1: 600), Akt (1: 400), p21 (1: 600), p27 (1: 600), or ABCAM (1: 600)). All antibodies were purchased from Abcam (Cambridge, UK). The washed membranes were incubated with the corresponding secondary antibodies.

The enhanced chemiluminescence (ECL) was used to visualize the results and the bands were developed using AlphaImager 2200. Data were adjusted according to the internal control (β-actin).

Data processing

Data are reported as mean ± SD (n = 3) and were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL). Student t-test was used to determine statistical significance with $p < 0.05$ considered statistically significant.

RESULTS

Effects of diosgenin on cell proliferation

Diosgenin's influence on primary human thyrocytes was examined: cell viability was not interrupted significantly when 15 µM of diosgenin was used for 24 h, while 20 µM of diosgenin inhibited cell proliferation ($p < 0.001$) – a concentration- and time-dependent manner was thus concluded (Figure 1).

EdU assay was used to examine the influence of diosgenin on cell proliferation. It was found that EdU-positive cells significantly reduced in number in the diosgenin-treated group (Figure 2 A), in an apparent concentration-dependent and time-dependent manner (Figure 2 B).
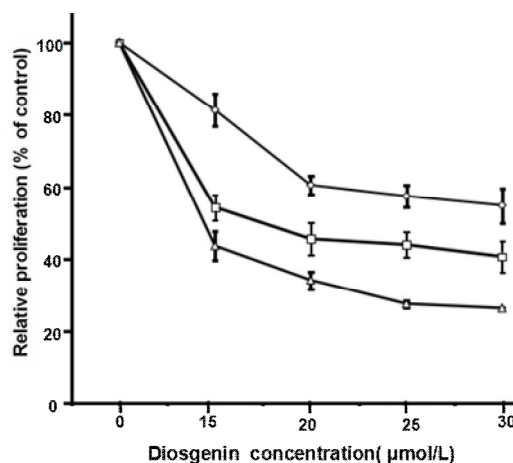

Figure 1: Influence of diosgenin on cell viability. Results were presented as ratio, relative to a negative control with culture medium only. ◊ = treatment for 24 h; □ = treatment for 48 h; Δ = treatment for 72 h
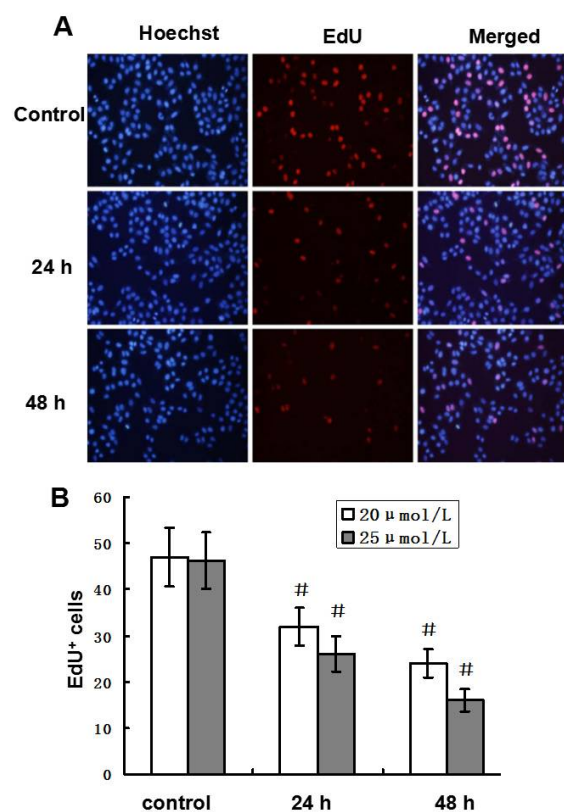

Figure 2: Diosgenin inhibited cellular DNA replication in primary human thyrocytes(HP: 200×). (A) Fluorescent staining showed incorporation of EdU in thyrocytes co-cultured with diosgenin for 24 and 48 h. (B) EdU-positive cells after 24/48 h of treatment was obtained using five consecutive visual fields ($p < 0.05$)

Influence of diosgenin on cell cycle analyzed by FACS

Thyrocytes were incubated in the growth medium supplemented with NBS (0.2 %) for 48 h in order to initiate quiescent after overnight starvation. 25

µmol/L diosgenin was added to the cells, which were left for 24, 48, and 72 h of incubation, respectively. More cells in G_0/G_1 phase and fewer in S phase were observed in diosgenin-treated groups compared to the control group. However, the number of cells in G_2/M phase remained unchanged (Figure 3, Table 1).
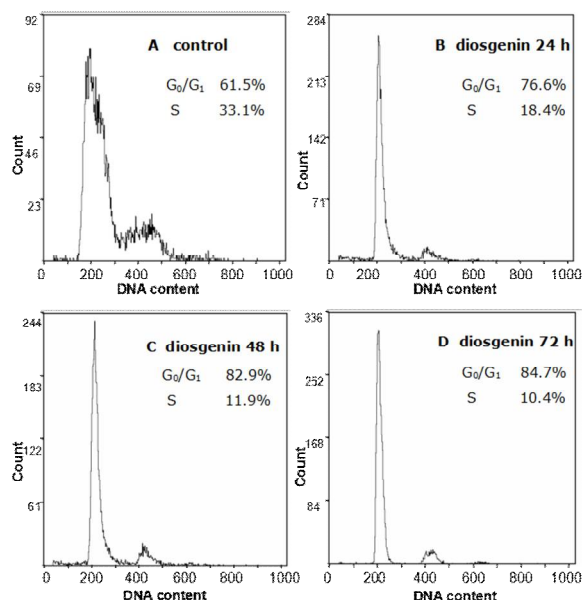

Figure 3: Effect of diosgenin on cell cycle. Thyrocytes were incubated (A) for 48 h in the control group or (B) were exposed to diosgenin (25 µM) for 12 h for (C) 24 h (D) for 48 h

Effect of diosgenin on protein expression

Western blot was used to examine diosgenin's influence on protein expression. Diosgenin (25 µM) was incubated with primary human thyrocytes for different periods of time. It was found that after 6, 12 and 24 h of diosgenin treatment, lower expression levels of PI3K, p-PI3K, and p-Akt protein, but higher expression levels of p21 and p27, than the control group were observed. However, the protein level of Akt remained unchanged (Figure 4 A). Interestingly, cyclin D1 protein expression was not disrupted by diosgenin treatment for 6 h, whereas a 12- or 24-h diosgenin treatment decreased the expression of cyclin D1 (Figure 4B). Protein levels of cyclin B1, ERK, p-ERK, MEK and p-

MEK were similar in each group after the diosgenin treatment (Figure 4 C).
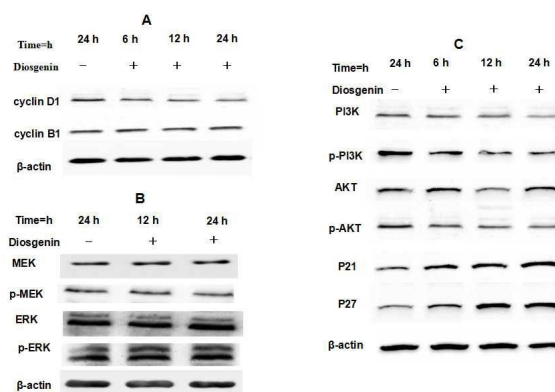

Figure 4: Effect of diosgenin on expression level of proteins. Thyrocytes were incubated with diosgenin (25 µM) (A, C) for 6, 12 or 24 h (B) were exposed to diosgenin (25 µM) for 12 or 24 h

DISCUSSION

In this study, inhibitory effects of diosgenin in primary human thyrocytes was examined and the possible mechanism for diosgenin action was investigated. It was found that the cell proliferation was halted upon addition of diosgenin and cell cycle was arrested at G_0/G_1, and that the anti-proliferative activity of diosgenin might involve the PI3K/Akt and MAPK signaling pathways.

Diosgenin was shown to inhibit the proliferation of primary human thyrocytes in both concentration- and time-dependent manners, consistent with the previous studies [15]. The potency of inhibition by the same concentration of diosgenin was however different in our experiments. This might be due to the cell type-specific responses to diosgenin.

Cell cycle is a common target of cancer therapies as it regulates the growth, proliferation, and death of cells, and is co-regulated by cyclins, cyclin-dependent kinases (CDKs) and their inhibitors. CDKs regulated transitions between cell cycle phases and are also involved in cell cycle arrest [16].

Table 1: Effect of diosgenin on cell cycle

Variable	Control	Diosgenin2 4 h	Diosgenin4 8 h	Diosgenin 72 h
G_0/G_1	61.5±5.8	76.6±4.6*	82.9±3.3*	84.7±4.4*
S	33.1±4.9	18.4±4.4*	11.9±2.1*	10.4±3.2*
G_2/M	5.4±0.5	5.0±1.6	5.2±1.8	5.1±0.4

* Compared with control ($p < 0.05$)

Our results demonstrated that diosgenin exposure triggered cell cycle arrest at G_0/G_1, as well as decreased expression of cyclin D1. Cyclin D1 acted as a rate-limiting component in cell cycle progression with which its expression varied whereas cyclin B1 regulated G_2/M checkpoint [17]. The research indicated that diosgenin arrested cell cycle via downregulating cyclin D1.

The anti-proliferative activity of diosgenin was shown by both arrest of cell cycle and trigger of apoptosis in some cancers [18]. In addition, cell cycle arrest is also associated with downregulation of TNF-induced expression of cyclin D1 [19]. Additionally, diosgenin arrested breast cancer stem-like cells in sub-G_1 phase [17]. Our results suggested that diosgenin arrested cell cycle of primary human thyrocytes by downregulating cyclin D1.

p21 and p27 prevent cell cycle progression by binding to cyclin-CDK complexes [20]. They are regarded as tumor suppressors as their upregulation inhibits proliferation of many cancer cells [21]. Diosgenin treatment increases expression of p21 and p27 in primary human thyrocytes, indicating that expression of p21 and p27 might associate diosgenin-induced cell cycle arrest.

PI3K/Akt signaling pathway was involved in biological processes such as metabolism, proliferation of cells and thyroid carcinogenesis [22]. Downregulation of the pathway has been reported to suppress cancer progression [23].

Inhibition of PI3K/Akt pathway by LY294002 upregulated expression of p21 and p27 [24] and caused to cell cycle arrest [25]. Our results showed that diosgenin treatment inhibited phosphorylation of Akt, but did not alter the expression levels of several proteins (MEK, p-MEK, ERK, p-ERK). The results suggest that deactivating PI3K/Akt pathway was involved in diosgenin-triggered cell cycle arrest in primary human thyrocytes, accompanied by an increase in the expression level of p21 and p27.

CONCLUSION

The findings of this work indicate that diosgenin treatment decreases proliferation of primary human thyrocytes and triggers G_0/G_1 arrest by downregulating cyclin D1. Deactivation of Akt and increased expression of p21 and p27 are involved in the anti-proliferative activitm y of diosgenin. Thus, diosgenin and its derivatives may have potentials for development as therapies for GD and other proliferative thyroid diseases.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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