Triptolide can Promote Cell Proliferation and Secretion in Primary Cultured Rat Adrenocortical Cells through pharmacologic serum treatment

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INTRODUCTION

Tripterygium wilfordii Hook f (TWHF) is a Chinese traditional medicine recorded in Compendium of Materia Medica, has much pharmacological activities of anti-inflammatory, immunoregulation, anti-tumor and anti-reproductive. TWHF has been first performed in rheumatoid arthritis and shows satisfactory effects in 1974. TWHF has excellent curative effect on glomerulonephritis in 1981. Lately, triptolide, a diterpenoid triepoxide, isolated from TWHF, has been widely performed in clinical treatment and shows satisfactory effects.

Triptolide alternatively combined with prednisone has been used in rheumatoid arthritis, asthma, nephrotic syndrome and shows satisfactory effects in clinical treatment. Triptolide, show slower effects than the hormone, but more durable and stable, has anti-inflammatory, immunoregulation, corticoid-like activities, but has no prednisone side effect. Triptolide enriches or replenishes the deficiency therapy of corticosteroids and show the best compatibility with alternatively combined use[1-3].

Clinical studies clues to triptolide has relation with the neuroendocrine system. In vivo experimental, we confirmed that triptolide has effects through the neuroendocrine system, especially through the hypothalamus-pituitary-adrenal axis (HPAA)[4-7]. However, it is very difficult to elucidate the details links of triptolide on each level of HPAA for complex nervous - endocrine - immune regulation in the internal environment.

In vitro, triptolide has been shown effects on smooth muscle cell[8], lung cancer cell[9] and astrocyte[10]. But, up to now, neither study on hypothalamus nor pituitary nor adrenal. We found triptolide can obviously promote corticosterone production and the fascicular zone cell proliferation in adrenal cortex in rat in vivo. So, in this study, we design to observe the effect of triptolide on adrenocortical cells in vitro. The present study will provides in vitro data and theoretical basis for comprehensive appraising the effects of triptolide, for reasonable, safe and efficient clinical practice of TWHF.

ABSTRACT

Objective: To study the effects of triptolide on cultured rat adrenocortical cells in vitro. Methods: To acquire pharmacologic serum, rats were randomly divided into five groups: the control group, the prednisone treated group, and the three groups treated with low, medium and high dose of the triptolide by gastrogavage. Primary adrenocortical cells were cultured and treated using different groups pharmacologic serum. 3β-HSD mRNA expression in adrenocortical cells were tested by Realtime - PCR, corticosterone levels were detected with ELISA, the expression of PCNA was examined by immunocytochemistry and the ultrastructure of adrenocortical cells was observed under electron microscope. Results: As compared with the control group, 3β-HSD mRNA expression and corticosterone raised significantly in all dose of the triptolide treated groups, while the prednisone treated group down-regulated. Medium and high dose of the triptolide treated groups show higher staining intensity of PCNA and the ultrastructure of adrenocortical cells appeared to change correspondingly. Conclusion: Triptolide exerted effects of stimulating on rat adrenocortical cells in vitro. Triptolide could promote the proliferation and secretion effect of adrenocortical cells in Primary cultured rat adrenocortical cells.

Keywords: Triptolide; prednisone; drug serum; primary culture; adrenocortical cells
MATERIALS AND METHODS

Drugs and Reagents

Triptolide (purity > 99%) was supplied by Department of Pharmacy, Zhongshan Hospital, Fudan University (Shanghai, China). The drug was initially dissolved in dimethyl sulfoxide (DMSO, 2% by final volume) and diluted with normal saline to the final concentration when used. Prednisone tablets was purchased from Shanghai Pharmaceutical (Group) Corporation (Shanghai, China), dissolved in normal saline.

Preparation of Pharmacologic Serum

Thirty adult healthy male SD rats (28-32 days, 180-220g) were randomly divided into control group, prednisone group (6mg/kg·d), high (600ug/kg·d), middle (400ug/kg·d) and low (200ug/kg·d) dose triptolide treated groups. Each group have six rats. Prednisone group and three doses of triptolide were administrated to rats by gastroavage two times per day for seven days; Control rats was similarly given an equivalent quantity of normal saline. One day after final administration, the rats were sacrificed under anaesthesia with pentobarbital. Vein blood was collected, centrifugated for collecting serum, then heated 56°C, 30min for deactivating complement. Serum samples were aliquoted and stored at -80°C for subsequent use.

Isolation and Culture of Adrenocortical Cells

Adult healthy male SD rats (28-32 days, 180-220g) were sacrificed under anaesthesia with pentobarbital. Two sides of adrenal glands were taken out following median laparotomy. The adrenal glands were free of adherent fat and medulla and minced into 1-mm pieces in Hank’s solution buffer, then resuspended with 5ml collagenase (2mg/ml) and DNase (0.2mg/ml) and incubated in a continuous shaker apparatus at 37°C for 40 min. Afterward, the suspension was filtered through a 125-mm nylon mesh, washed twice with HBSS, 1000×g for 10 min. Cells were collected and seeded in 35mm dishes in incubator (37°C, 5% CO2, 95% O2, pH 7.4±0.2). Cell density was adjusted to 2.5×10^5 cells per dish. Culture medium contains 20% fetal calf serum, 100m units/ml penicillin G, 100mg/ml streptomycin sulfate and L-glutamine. Cells and medium were collected after culture for the following experiments.

Histological Analysis and Identification of Adrenocortical Cells

Culture adrenocortical Cells were identified using O’Hare method for histochemical staining of 3β-hydroxysteroid dehydrogenase (3β-HSD) combined with light microscopic observation[11].

Immunocytochemistry Staining of PCNA

The paraformaldehyde-fixed cells in chamber slides were washed with PBS solution, endogenous peroxidase activity was blocked by a 30 min incubation in 3% hydrogen peroxide/methanol buffer. The slides were next incubated with 5% BSA/TBS for 20 min at room temperature to reduce nonspecific background staining. A primary monoclonal anti-PCNA antibody at a 1:100 dilution (Dako Cytomation, Carpinteria, CA) were applied for overnight at 4°C in TBS. After a series of TBS rinses, secondary antibody was detected by using an anti-mouse horseradish peroxidase-labeled polymer secondary antibody from the EnVision system (Dako). The slides were rinsed in TBS and visualized by DAB in buffered substrate (Dako). Finally, the slides were counterstained with hematoxylin. In negative controls experiments, BSA was used and the primary antibodies were omitted. The positive result showed brown coloration in the nucleus. The percentage of PCNA positive cells was calculated. The PCNA positive cell number/total cell number ratio was calculated in each of 10 fields under light microscope. The total positive expression rates were measured.

Transmission Electron Microscopy Observation

Cultured adrenocortical cells were prefixed at 4°C for 2h in 4.5% glutaraldehyde buffered with 0.1M sodium cacodylate-HCl at pH 7.4 and postfixed for 24h in 2% osmium tetroxide, then processed with the standard technique.

Corticosterone ELISA

Culture medium concentration of corticosterone was determined using commercially available ELISA kits (Diagnostic Systems Laboratories, USA). Each 25ul of standards, controls and culture mediums were added to the appropriate wells, then100ul of the rat corticosterone enzyme conjugate solution and rat corticosterone antiserum were added to each well. After 1h incubation, the wells were washed and developed using TMB chromogen solution. Absorbance was read at 450 nm and medium concentration values were calculated from the standard curve.
RNA Isolation and Real-time-PCR

Total RNA was isolated from cultured adrenocortical cells using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer’s recommendations. The concentration of RNA samples was ascertained by measuring optical density at 260 nm. The quality of RNA was confirmed by the detection of 18S and 28S bands after agarose gel electrophoresis. Total RNA was used to generate cDNA using a MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Invitrogen Inc., Carlsbad, CA) with oligo (dT) primers, according to the manufacturer’s protocol. The RT reactions were carried out at 37 °C for 15 min followed by a 5-sec denaturation step at 85 °C. And then, real-time quantitative PCR amplification reactions were carried out in an ABI Prism 7500 sequence detection system (Applied Biosystems) in a 20ul volume for the detection of 3β-HSD and β-actin which was used as a reference gene using SYBR Premix Ex Taq™ Kit (TaKaRa Bio Inc.), according to the manufacturer’s protocol. The reaction mixture containing: 10ul SYBR Premix Ex Taq(2×), 0.2uM of each primer, 0.4ul ROX Reference Dyel, 50ng cDNA template. The PCR conditions were: 95 °C/30 sec, annealing temperature/5 sec, 60 °C/34 sec, 40 cycles and 95 °C/15 sec, 60 °C/60 sec, 95 °C/15 sec. The primers and the annealing temperatures were as follows: 3β-HSD, sense: CTCCATGCCAAACCAGTG, anti-sense: AGGAAGGTCCCAAACCTGAAT (60 °C); β-actin, sense: GGAAATCGTGCGTGACATT, anti-sense: CGGCCAGTGCCCATCTC (60 °C). Each sample was run in triplicate, and averaged triplicates were used to assign cycle threshold (CT) values.

Statistical Analysis

Statistical significance of comparison of biochemical parameters was determined by calculation of p-values using SPSS 23 statistics software (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphological Observation and Identification of Adrenocortical Cell

Primary cultured adrenocortical cells can adhere to the wall and slowly growth. Cells initially show clear spherical nuclei and large polygonal shape cytoplasm, having small granules in cytoplasm. Along with the culture and passage, cells gradually changed into spindle shape. Histochemical staining of 3β-HSD show adrenocortical cells have positive, violet-blue granule sediments in cytoplasm (Figure 1).

PCNA staining density in cultured adrenocortical cells PCNA positive staining show brown color localized in the nuclei of cultured adrenocortical cells. PCNA density was evaluated as the labeling index (LI). The LI was the highest in the TPM and TPH group, intermediate in the control group, and lowest in the prednisone group (p < 0.05) (Table 1).

Comparison of Corticosterone Concentrations by ELISA

Corticosterone concentrations increased in all three triptolide treated groups and decreased in the prednisone treated groups compared with the controls (p < 0.05). Compared with prednisone treated groups, corticosterone concentrations increased in all three triptolide treated groups (p < 0.05). Analysis of variance indicated that significant differences of corticosterone were respectively found among the groups (p < 0.05). In three triptolide treated groups, corticosterone concentrations in TPM and TPH groups is lower than TPL group (p < 0.05) (Table 2).

3β-HSD Gene Expression Detected by Real Time-PCR

Our results show that 3β-HSD mRNA expression of all three triptolide treated groups were significantly upregulated (p<0.01) while prednisone treated groups downregulated (p<0.01) compared with control groups (Figure 3).

DISCUSSION

In clinical, both Tripterygium wilfordii Hook f and corticosteroids have a dual actions of anti-inflammatory and...
immunodepression function, and applied for the treatment of a wide range of autoimmune diseases. Clinical studies revealed that although long-term intake of prednisone can effectively control the pathogenetic conditions, but can inhibit adrenal cortex function, which in turn cause a significant decline of blood hydrocortisone concentration, followed by different degrees of Cushing syndrome and may complication with diabetes[12]. Tripterygium wilfordii polyglycosidium (TWP) such as triptolide features as anti-inflammatory, immunosuppressive and corticoid-like actions while have no side effects of prednisone. The usage of TWHF can withdrawal or significantly reduce the application of prednisone. Therefore, it can avoid side effects of taking long-term hormones, but also aim to achieve an effective treatments.

However, experimental and clinical studies remain incomplete as to the mechanisms and impacts of triptolide’s corticoid-like anti-inflammatory actions. Apparently this incompletion objection a more safe and reasonable usage of triptolide in clinical treatment.

In the previous study, using histology, histochemistry, electron microscopy and radioimmunoassay methods, we discovered that extractives of Tripterygium wilfordii Hook f can promote the function and activity of adrenal cortex, especially fascicular zone cells, which in turn stimulates their proliferation and secretion of adipoid and corticosterone. we have confirmed the promote effect of triptolide on the hypothalamic-pituitary-adrenal axis(HPAA) in vivo[4-7]. But it is hard to explain precisely the target link in triptolide’s action on HPAA and the mechanisms of its anti-inflammatory and immunomodulation functions.

In this study, we use in vitro cell culture methods to separate adrenocortical cells from rats to obviate the influence of internal environment, such as HPAA, which eliminating impacts of hypothalamic and pituitary. To mimic the pharmacologic actions of triptolide in vivo, we use drug serum to cultivate adrenocortical cells and observe the effects of triptolide on cells.

Japanese scholars Iwama first developed the drug serum method in their study of traditional Chinese medicines[13]. Drug serum may contain ingredients which play direct functions of the drug as well as various metabolized products of endogenous substances induced by the drug. So, drug serum can explain the mechanisms of pharmacological actions of traditional Chinese medicine within the body more objectively and correctly. In our study, adrenocortical cells were treated with triptolide serum in vitro, and by testing the corticosterone concentrations, significant increases

<table>
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<tr>
<th>Group</th>
<th>Corticosterone(ng.ml⁻¹)</th>
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<tbody>
<tr>
<td>Control</td>
<td>144.27±13.58</td>
</tr>
<tr>
<td>Prednisone</td>
<td>120.11±14.35</td>
</tr>
<tr>
<td>TPL</td>
<td>205.03±10.02*</td>
</tr>
<tr>
<td>TPM</td>
<td>172.87±8.16**</td>
</tr>
<tr>
<td>TPH</td>
<td>167.78±11.01**</td>
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</tbody>
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*Significantly different from the control group, p < 0.05.
†Significantly different from the prednisone group, p < 0.05.
*Significantly different from the TPL group, p < 0.05
was observed in all the triptolide groups compared with the control groups, while obvious decreases took place in the prednisone groups. The results revealed that direct promote effects of triptolide on adrenocortical cells in terms of biological functions, which in turn played crucial role in triptolide treatment.

PCNA is a nucleus biomarker of cell proliferation, which is indispensable to DNA synthesis and is a good indicator of cell proliferation activity[14]. In this study, the levels of staining density of PCNA, in medium and high dose of triptolide treated groups was significantly stronger than the control groups, while weaker in the prednisone treated groups, which illustrates the stimulus function of triptolide on the proliferation of adrenocortical cells in terms of morphology. These results correspond to the observed functional changes of adrenocortical cells. In adrenocortical cells, mitochondria and SER and ARE are the main subcellular structures participating in steroid hormone synthesis[15]. As a result, the changes in their quantity and morphology should reflect the function of adrenal cortex. Abundant mitochondria and SER were observed in three prednisone treated groups, showing active function of the adrenal cortex. On the contrary, the prednisone treated groups showing opposite results. These findings prove the promote effects of triptolide on adrenal cortex in terms of ultrastructure.

3β-HSD, as a key enzyme turning ketopregnene into progesterone, which can in turn be transformed into corticosterone, cortisol and androsterone under the catalysis of coherent enzymes, mainly exists in steroid hormones secreting cells [16]. In this study, we detect 3β-HSD mRNA levels by real time-PCR as an indicator of synthetic function of these cells. 3β-HSD mRNA in all three triptolide treated groups with different doses were significantly higher than the control groups, while lower in prednisone treated groups, indicating stimulus function of triptolide on the adrenal gland.

In conclusion, our observations in terms of function, morphology and molecular biology make it clear that triptolide bears a direct stimulus function on adrenal gland, by promoting the proliferation of adrenocortical cells and stimulating the secretion of corticosterone, and apparently the mechanism is different from that of prednisone. But does triptolide exerts only on the adrenal gland and in turn on the HPAA axis? Henceforth we intend to study the influences of triptolide on rats’ hypothalamus tissue and pituicytes as well as the mechanisms in vitro, and to conduct multi angle explorations into the target organs, tissues and cells of triptolide within the HPAA axis as well as the mechanisms of its corticoid-like anti-inflammatory and immunosuppressive actions.

REFERENCES