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Comparison of natural estrogens and synthetic derivative on genioglossus function and estrogen receptors expression in rats with chronic intermittent hypoxia

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ABSTRACT

The pathogenesis of obstructive sleep apnea–hypopnea syndrome (OSAHS) is summarized as the narrow anatomic structure of upper airway (UA) and the defective function of UA dilator muscles. Up to now, there have been no specific treatments for the UA dilator muscle deficiency. We previously found that some estrogen-like compounds exert protective effects on genioglossus, but this protection tends to be less satisfactory. A novel phytoestrogen derivative was synthesized in recent years and was verified to have some cytoprotective activity. This study was designed to compare the effects of natural estrogens and the synthetic resveratrol dimer on genioglossus contraction and expression of estrogen receptors (ERs) under chronic intermittent hypoxia (CIH) condition. Genioglossus myoblasts of rat were isolated and cultured in a culture medium with different agents (estradiol, genistein, resveratrol, and resveratrol dimer, respectively) under hypoxia condition, and ERs expressions were detected. In vivo study, 48 ovariectomized female rats were randomized into six groups. After CIH exposure and agents injection, rats were tested for genioglossus contractile properties and further analysis of ERs expression. Estradiol up-regulated ERα level and exerted the best protective effect of fatigue resistance. Genistein, resveratrol and resveratrol dimer primarily up-regulated the expression of ERβ. Resveratrol dimer exhibited better protection of fatigue resistance than genistein and resveratrol, and expressed higher binding affinity for ERβ than for ERα. Besides estrogenic effects, there may be some other mechanisms for the fatigue resistance improvement contributed by phytoestrogens and their derivatives.

1. Introduction

Obstructive sleep apnea–hypopnea syndrome (OSAHS) is a chronic disorder characterized by intermittent and recurrent upper airway (UA) obstruction during sleep, associated with hypoxia and hypercapnia which have deleterious consequences on physical health and quality of life [1]. Untreated OSAHS increases the risk of cardiovascular diseases, neurocognitive dysfunction, metabolic disturbances and even sudden death [2–4]. The pathogenesis of OSAHS is multifactorial. The main cause of OSAHS consists of the narrow anatomic structure and the defective function of UA. In comparison with normal people, patients with OSAHS have narrower airways and decreased muscle tone during sleep. The state of UA depends on the balance between the positive intraluminal pressure to open the airway and the negative surface tension to keep it closed [1]. Therefore, UA dilator muscles may be the key point in the study of pathogenesis and therapy of OSAHS. Genioglossus, an important UA dilator muscle, is the main tongue muscle which exerts forward propulsion to the tongue and contracts in coordination before the diaphragm contracts. As for the important role of genioglossus in maintaining UA patency, it is called as the safe guard of the UA [5].

Chronic intermittent hypoxia (CIH), with an interpretation "repetitive hypoxia interspersed with episodes of normoxia" [6], plays a major role in causing many abnormalities associated with OSAHS. Exposure to CIH increases the activation of the UA dilator muscles, causes a transition from slow to fast in muscle fiber types [7], and produces a widespread reduction in endurance [8]. In our previous studies, estradiol has been verified in protection on CIH damage and improvement of the fatigue resistance of genioglossus in ovariectomized rats [9–11]. However, the clinical applications of estradiol are restricted because of the serious side effects such as cardiovascular diseases and breast cancers [12]. Therefore, substitutes for estrogen are needed to avoid the undesirable systemic complications.

Phytoestrogens, derived from a variety of plants, are a diverse of non-steroidal and estrogen-like compounds including the isoflavonoids (genistein and daidzein), the flavonoids (kaempferol

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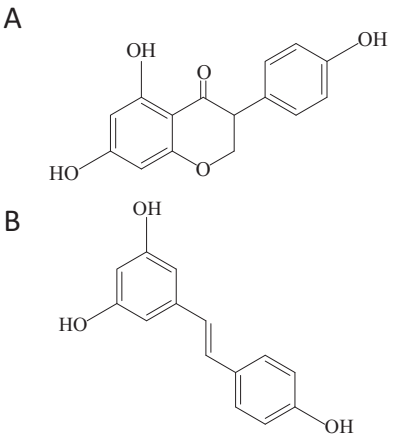

Fig. 1. Chemical structures of the natural phytoestrogens used in this study: (A) genistein, (B) resveratrol.

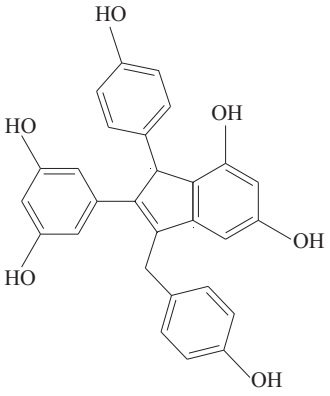

Fig. 2. *Endo*-shifted olefin isomer of parthenocissin A (Resveratrol dimer used in this study).

and quercitin), the lignans (enterolactone and enterodiol), the stilbens (resveratrol), the coumestanes (coumestrol), and the mycotoxins (zearalenol) [13]. Phytoestrogens exert estrogen and antiestrogen effects via binding to estrogen receptors (ERs), carrier proteins and other molecules, directly or indirectly involved in the estrogenic signal transduction. These estrogenicity and antiestrogenicity of phytoestrogens result from some structural similarity with estrogens: the phenolic hydroxyls at their extremities [14]. Because of less side effects and long-term health benefits (prevention of osteoporosis, cardiovascular disease and breast cancer) [15], phytoestrogens may be an alternative of estrogens to postmenopausal hormone therapy and other therapies.

With similarity to estrogen, phytoestrogens mimic the actions of mammalian estrogens primarily through binding to ERs [16]. ERs consist of two subtypes, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which differ from each other in C-terminal ligand-binding domain and N-terminal transactivation domain [17]. ERs possess common structures including five functional domains: A/B, C, D, E and F, contributing to transcriptional activation, DNA binding, nuclear translocation, ligand binding and so on, respectively [18,19]. Both ERα and ERβ belong to the nuclear receptor family of ligand-dependent transcription factors, mediate genomic and nongenomic events and trigger a series of tissue-specific responses. ERα and ERβ mediate distinct biological effects partly because of the differences in tissue distribution and expression level. Kuiper found in 6- to 8-week-old rats moderate to high expression of ERα in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal, and abundant expression of ERβ in prostate, ovary, lung, bladder, brain, uterus, and testis [20].

Genistein (Fig. 1A), a member of the isoflavonoids, is one of the most extensively studied phytoestrogens. Genistein exerts estrogenic as well as anti-estrogenic actions with tissue specificity and dose-dependent activity. Resveratrol (Fig. 1B), *trans*-3,5,4′-trihydroxystilbene, is a polyphenol found naturally in series of plants such as grapevines and characterized as a kind of phytoestrogen with abilities of binding to ERs and activating estrogenic effects. Sun and co-workers reported a novel approach to the synthesis of a series of resveratrol derivatives [21]. Among those derivatives, the resveratrol dimer showed in Fig. 2, is an *endo*-shifted olefin isomer of parthenocissin A, which acted as a synthetic selective estrogen receptor modulators (SERMs) in pre-experiment.

In present study, we hypothesized that resveratrol dimer might exert a more significant role in genioglossus fatigue resistance. We aimed to make a comparison on genioglossus function and estrogen receptor binding affinity among natural estradiol, natural phytoestrogens and synthetic phytoestrogen derivative to analyze the inner mechanism of these effects.

2. Materials and methods

2.1. Materials

Both neonatal and adult Sprague–Dawley (SD) rats were from Experimental Animal Center of Second Military Medical University (Shanghai, China). Estradiol was obtained from Sigma-Aldrich (St Louis, MO, USA). Genistein and resveratrol were from Tauto Biotech (Shanghai, China). Resveratrol dimer was obtained from Professor Sun and co-workers (School of Pharmacy, Fudan University, Shanghai, China). MTT cell proliferation and cytotoxicity detection kit and BCA protein assay kit were from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Cell culture of genioglossus myoblasts

Under sterile conditions, muscle tissue of genioglossus from 2–3-day-old rat was excised, minced, separated from connective tissue, and rinsed with Hank's solution. After transferred to a centrifuge tube, the muscle slurry was digested with 0.05% collagenase type II at 37 °C for 40 min with continuous slow shaking, and centrifuged at 1000 rpm for 1 min. Further digestion was initiated in 0.25% trypsin-EDTA at 37 °C for 30 min, and stopped by the addition of 20% fetal bovine serum (HyClone, USA). After that, cells were passed through a 75 μm sieve (Millipore, USA), centrifuged at 1000 rpm for 1 min, resuspended in DMEM supplemented with 25% fetal bovine serum, and plated on culture dishes after twice repeatedly differential attachment treatment. After reaching 80% confluence, the growth medium was replaced with normal medium (10% fetal bovine serum in DMEM).

Normoxic genioglossus myoblasts were cultured at atmospheric oxygen concentration (21% O_2, 5% CO_2; balance N_2) for 24–72 h in incubator. Myoblasts exposed to hypoxia were cultured in a hypoxia chamber (1% O_2, 5%CO_2; balance N_2) for the same length of time.

2.3. MTT assay of resveratrol dimer

An MTT assay was used to determine the influence of the resveratrol dimer on proliferation of genioglossus myoblasts. Cells (3×10^3/well) were plated in 100 μL medium/well and inoculated in 96-well plates for 24 h. Then the culture medium of experimental group was replaced with 100 μL resveratrol dimer-containing medium of concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} mol/L. In the control group, culture medium was refreshed