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Effects of resveratrol on rat neurosteroid synthetic enzymes

Yiluan Wang[a,1], Jianliang Sun[a,1], Ling Chen[b], Songyi Zhou[a], Han Lin[a], Yiyan Wang[a], Nengming Lin[b,*], Ren-Shan Ge[a,*]

[a] Department of Anesthesiology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027, China
[b] Translational Medicine Research Center, Affiliated Hangzhou Hospital (Hangzhou First People's Hospital), Nanjing Medical University, No. 261 Huansha Road, Hangzhou, Zhejiang 310006, China

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ABSTRACT

Resveratrol, a common polyphenol, has extensive pharmacological activities. Resveratrol inhibits some steroid biosynthetic enzymes, indicating that it may block neurosteroid synthesis. The objective of the present study is to investigate the inhibition of resveratrol on neurosteroidogenic enzymes rat 5α-reductase 1 (SRD5A1), 3α-hydroxysteroid dehydrogenase (AKR1C9), and retinol dehydrogenase 2 (RDH2). The IC_{50} values of resveratrol on SRD5A1, AKR1C9, and RDH2 were > 100 μM, 0.436 ± 0.070 μM, and 4.889 ± 0.062 μM, respectively. Resveratrol competitively inhibited rat AKR1C9 and RDH2 against steroid substrates. Docking showed that resveratrol bound to the steroid binding pocket of AKR1C9. It exerted a mixed mode on these AKR1C9 and RDH2 against cofactors. In conclusion, resveratrol potently inhibited rat AKR1C9 and RDH2 to regulate local neurosteroid levels.

1. Introduction

Resveratrol, chemically called 3,4′,5-trihydroxy-trans-stilbene, is a polyphenol extracted from food, such as grape skins, peanuts, red wine, and berries. Lots of attentions have been paid to this chemical because it displays diverse pharmacological activities, such as protective effects against the cardiovascular diseases [1], anti-diabetic effects [2], anti-inflammatory or anti-carcinogenic activities [3], and anti-aging properties [4]. It was also reported that resveratrol prevented high-fat-diet induced metabolic syndrome in a mouse model [2] possibly due to the fact that it can activate PPARγ receptor [5]. It has been shown to be an anti-aging agent of a short-lived fish and other animals [4]. In addition, resveratrol exhibited the neuroprotective effects in cells of rodent models [6–9].

In our previous study, we found that resveratrol inhibited steroid biosynthesis production in rat Leydig cells by directly blocking 3β-hydroxysteroid dehydrogenase [10], indicating that resveratrol may disturb the synthesis of other steroids, such as brain neurosteroids.

Neurosteroids are a class of steroids that have neurological activities. These steroids include allopregnanolone (ALLO) and 5α-androstanediol (DIOL) in the brain. Usually, steroids bind to the nuclear receptors, the transcription factors, to take their action [11]. Instead of the nuclear receptors, neurosteroids bind to the membrane receptor such as $GABA_A$ receptors as the agonists to regulate neural functions [12]. When the brain ALLO and DIOL levels were increased, they cause anxiolytic, anticonvulsant, analgesic, and sedative effects [12].

The synthesis of ALLO and DIOL requires brain steroid 5α-reductase (EC 1.3.99.5) and 3α-hydroxysteroid dehydrogenase (AKC1C9, EC 1.1.1.50) (Fig. 1). Steroid 5α-reductase is a microsomal NADPH-dependent enzyme. Two enzymes responsible for 5α-reductase activity have been identified: type I (SRD5A1) and type II (SRD5A2), encoded by two genes, *Srd5a1* and *Srd5a2*, respectively [13]. Both enzymes convert the same reaction but at different pH optima and different substrate affinities, with SRD5A1 of a broad optimal pH range of 6.0–8.5 and a Km of 1.0–5.0 μM and SRD5A2 of a narrow acidic optimal pH of 5.5 and a Km of 0.1–1.0 μM [13]. Both enzymes are expressed in the brain, with SRD5A1 in a broad brain region and throughout the development and adulthood and SRD5A2 in the limited brain region during a restricted perinatal period in rodents [14]. Therefore, in the present study, we focused on SRD5A1. SRD5A1 catalyzes a number of Δ4,3-ketosteroids (progestins, androgens, and glucocorticoids) to their 5α-reduced metabolites. In the brain, it primarily converts either testosterone into dihydrotestosterone or progesterone into dihydroprogesterone [15]. Once the above irreversible conversion takes place, the reduced 5α-reduced metabolites can be further converted into the neurosteroid DIOL or ALLO via AKR1C9 (encoded by *Akr1c9*). AKR1C9 is a cytosolic NADPH-dependent enzyme (Fig. 1). In the brain, highest levels were found in the olfactory bulb and moderate levels

* Corresponding authors.
E-mail addresses: lnm1013@163.com (N. Lin), renshan_ge@wmu.edu.cn (R.-S. Ge).
[1] These two authors contributed to this work equally.

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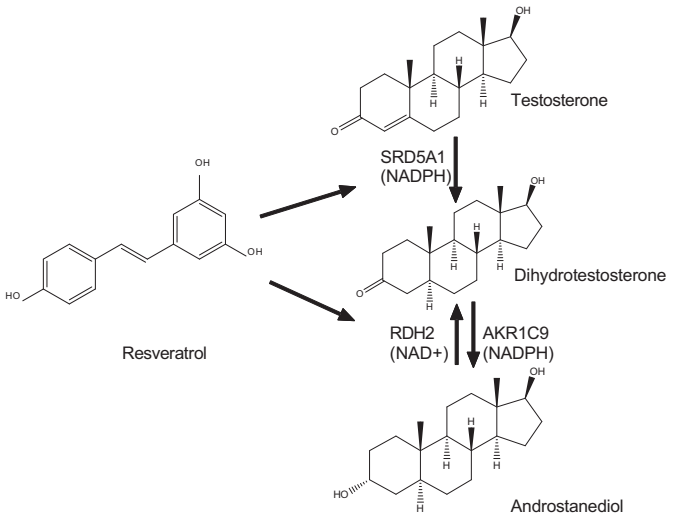

Fig. 1. The biosynthesis and metabolism of neurosteroids, allopregnanolone and androstanediol, by three distinct enzymes: NADPH-dependent 5α-reductase 1 (SRD5A1), NADPH-dependent cytosolic 3α-hydroxysteroid dehydrogenase (AKR1C9), and NAD^+-dependent microsomal retinol dehydrogenase 2 (RDH2).

were found in the cerebellum, cerebral cortex, hypothalamus, and pituitary [16]. Interestingly, retinol dehydrogenase 2 (RDH2, encoded by *Rdh2*), another brain microsomal NAD^+-dependent enzyme, not only catalyzes the retinol metabolism but also catalyzes the opposite direction of AKR1C9 (Fig. 1) [17]. Thus, RDH2 also regulates the levels of ALLO or DIOL [18]. However, whether resveratrol can directly interact with these neurosteroidogenic enzymes is still unclear. In the present study, we investigated the direct inhibitory effects of resveratrol on these three neurosteroidogenic enzymes.

2. Materials and methods

2.1. Materials and chemicals

[^{3}H]Dihydrotestosterone, [^{3}H] androstanediol and [^{3}H] testosterone were obtained from DuPont-New England Nuclear (Boston, MA). Dihydrotestosterone, androstanediol, and testosterone were purchased from Steraloids (Newport, RI). Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO). Resveratrol was dissolved in dimethyl sulfoxide. Rat SRD5A1 gene *Srd5a1* or RoDH2 gene *Rodh2* in the pcDNA3.1 vector was cloned and rat AKR1C9 gene *Akr1c9* in the pRc/CMV vector was a gift from T M Penning (University of Pennsylvania, Philadelphia, PN). COS-1 cell line was purchased from ATCC (Manassas, VA). DMEM was purchased from Life Technologies Inc. (Gaithersburg, MD).

2.2. Transient transfection of Srd5a1, Akr1c9, and Rdh2

COS-1 cells were cultured in DMEM medium with 10% fetal bovine serum at 5% CO2 and 37 °C. Cells (1×10^6) were seeded per well in the six-well plate and cultured for 24 h in media added with charcoal-stripped fetal bovine serum and were maintained for 50–80% confluence. We transiently transfected *Srd5a1*, *Akr1c9*, and *Rdh2* into COS-1 cells, respectively, using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. We selected 1 μg DNA plasmid per well for the transfection because it was shown that maximal efficiency could be achieved by 1 μg DNA plasmid, and this quantity was used in all transfection assays.

2.3. Preparation of SRD5A1, AKR1C9, and RDH2 subcellular fractions

Twenty-four hours after transient transfection, we washed the transfected COS-1 cells with phosphate buffered saline three times, and scraped the cells using the rubber policeman, and transferred them into a glass homogenizer. 0.01 mM ice-cold phosphate-buffered saline containing 0.25 M sucrose was added to the homogenizer, and cells were gently homogenized in the ice-cold bath. Homogenates were transferred into a 15-ml centrifuge tube and centrifuged at 1500 × *g* for 10 min to remove the cell debris and nuclear fragments. The post-nuclear supernatants were transferred to a new tube and centrifuged at 10,000 × *g* (4 °C) for 30 min to remove the mitochondria. The supernatants were further transferred into a new ultra-centrifuge tube and centrifuged at 105,000 × *g* (4 °C) for 1 h twice, and the resultant microsomal pellets and cytosols were collected. We measured the protein concentrations of these subcellular fractions using a protein concentration assay kit (No. 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard. The concentrations of rat SRD5A1, AKR1C9, and RDH2 proteins were diluted to 20 mg/ml. The proteins were used for the measurement of SRD5A1, AKR1C9, and RDH2 activities. The activities of SRD5A1, AKR1C9, and RDH2 in the subcellular preparations were determined as follows.

2.4. Measuring SRD5A1, ARR1C9, RDH2 activities

SRD5A1 activity was measured using radiolabeled testosterone. Briefly, we incubated 1 μM testosterone spiked with 60,000 dpm [^{3}H]-testosterone(specific activity = 80.4 Ci/mmol), 10 μg SRD5A1-containing microsomes, and 0.2 mM NADPH in 250 μl phosphate buffered saline (pH = 7.2) at 37 °C from 15 min to 60 min. AKR1C9 activity was measured using radiolabeled dihydrotestosterone. We incubated 1 μM dihydrotestosterone spiked with 30,000 cpm [^{3}H]-dihydrotestosterone (specific activity = 90.0 Ci/mmol, counter efficiency is about 50%), 10 μg AKR1C9-containing cytosolic protein, and 0.2 mM NADPH in 250 μl phosphate buffered saline (pH = 7.2) at 37 °C from 15 min to 60 min. RDH2 activity was measured using radiolabeled androstanediol. We incubated 1 μM androstanediol spiked with 30,000 cpm [^{3}H]-androstanediol, 10 μg RDH2-containing microsomal protein, and 0.2 mM NAD^+ in 250 μl phosphate buffered saline (pH = 7.2) at 37 °C from 15 min to 60 min. We selected the reaction time range, in which SRD5A1, AKR1C9, and RDH2 velocities were linear. Resveratrol was dissolved in dimethyl sulfoxide as the stock solution of 25 mM, of which 1 μl was added to the above 250 μl phosphate buffered saline for the initial inhibition test (the final concentration of resveratrol was 100 μM). The inhibitory potency of resveratrol was calculated relative to control (only dimethyl sulfoxide). Resveratrol was dissolved in dimethyl sulfoxide at a final concentration of 0.4%, at which concentration dimethyl sulfoxide did not inhibit SRD5A1, AKR1C9, and RDH2 activities. At the end of incubation, the reaction was stopped by adding 2 ml ice-cold ether into the tube. The tubes were capped and vigorously vortexed for 1 min at room temperature. The tubes were centrifuged at 1500 × *g* for 5 min. The organic ether layer containing steroids was transferred into a new glass tube and dried up under nitrogen. The pellet in the glass tube was dissolved in 75 μl ether and applied onto a thin layer plate (Baker-flex, Phillipsburg, NJ), which was put into a tank containing chloroform and methanol (90:3, v/v) for 45 min. The thin layer plate was dried up in the holder. The radioactivity was measured by a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC) and the peak cpm values of the substrate and the product were recorded. The percentage conversion of testosterone into dihydrotestosterone (for SRD5A1), dihydrotestosterone into androstanediol (for AKR1C9), or androstanediol into dihydrotestosterone (for RDH2) was calculated by dividing the radioactive counts identified as the respective steroids by the total counts to calculate their activities as previously described [19].

2.5. Measuring enzyme kinetics

Apparent Michaelis constant (Km) and maximum velocity (Vmax)