Steroids 77 (2012) 1327–1334

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Structure–activity relationship (SAR) analysis of a family of steroids acutely controlling steroidogenesis

Andrew Midzak, Georges Rammouz [1], Vassilios Papadopoulos *

The Research Institute of the McGill University Health Centre, Departments of Medicine, Biochemistry, and Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1A4

ARTICLE INFO

Article history:
Received 28 June 2012
Received in revised form 25 August 2012
Accepted 28 August 2012
Available online 15 September 2012

Keywords:
Steroidogenesis
TSPO
Cholesterol
CRAC
Androstenetriol

ABSTRACT

Steroids metabolically derive from lipid cholesterol, and vertebrate steroids additionally derive from the steroid pregnenolone. Pregnenolone is derived from cholesterol by hydrolytic cleavage of the aliphatic tail by mitochondrial cytochrome P450 enzyme CYP11A1, located in the inner mitochondrial membrane. Delivery of cholesterol to CYP11A1 comprises the principal control step of steroidogenesis, and requires a series of proteins spanning the mitochondrial double membranes. A critical member of this cholesterol translocation machinery is the integral outer mitochondrial membrane translocator protein (18 kDa, TSPO), a high-affinity drug- and cholesterol-binding protein. The cholesterol-binding site of TSPO consists of a phylogenetically conserved cholesterol recognition/interaction amino acid consensus (CRAC). Previous studies from our group identified 5-androsten-3β,17,19-triol (19-Atriol) as drug ligand for the TSPO CRAC motif inhibiting cholesterol binding to CRAC domain and steroidogenesis. To further understand 19-Atriol's mechanism of action as well as the molecular recognition by the TSPO CRAC motif, we undertook structure–activity relationship (SAR) analysis of the 19-Atriol molecule with a variety of substituted steroids oxygenated at positions around the steroid backbone. We found that in addition to steroids hydroxylated at carbon C19, hydroxylations at C4, C7, and C11 contributed to inhibition of cAMP-mediated steroidogenesis in a minimal steroidogenic cell model. However, only substituted steroids with C19 hydroxylations exhibited specificity to TSPO, its CRAC motif, and mitochondrial cholesterol transport, as the C4, C7, and C11 hydroxylated steroids inhibited the metabolic transformation of cholesterol by CYP11A1. We thus provide new insights into structure–activity relationships of steroids inhibiting mitochondrial cholesterol transport and steroidogenic cholesterol metabolic enzymes.

1. Introduction

Steroid hormones regulate a wide variety of physiological and developmental processes in vertebrates. These lipid hormones are synthesized in the testes, ovaries, and adrenal cortex by the successive enzymatic transformation of the cyclopentanophenanthrene ring structure of cholesterol, the metabolic precursor of all steroids [1]. In these tissues, synthesis of cyclic adenosine monophosphate (cAMP), in response to stimulation by circulating peptide hormones, promotes the traffic of cholesterol from cellular stores to the inner mitochondrial membrane [2]. There, cholesterol is metabolized to the steroid pregnenolone by the cytochrome P450 enzyme CYP11A1, which hydroxylates and cleaves cholesterol's aliphatic tail [3]. Pregnenolone undergoes further metabolic processing by mitochondrial and endoplasmic reticulum cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes to generate the repertoire of steroid hormones [1]. The primary site of physiological control for steroidogenesis is the transport of cholesterol across the double membranes of the mitochondria to CYP11A1 in the matrix side of the inner mitochondrial membrane. A number of proteins have been found to be critical for this process, key among them the mitochondrial 18-kDa translocator protein (TSPO) [4,5].

TSPO is a phylogenetically conserved mitochondrial protein originally identified through its ability to bind benzodiazepines, and hence originally named the peripheral benzodiazepine receptor (PBR) [6,7]. Containing five alpha helices, TSPO spans the outer

Abbreviations: 19-Atriol, 5-androsten-3b,17,19-triol; 22R-HC, 22R-hydroxycholesterol; cAMP, cyclic adenosine monophosphate; CRAC, cholesterol recognition/interaction amino acid consensus; CYP11A1, cytochrome P450 11A1; dbcAMP, dibutyryl cyclic adenosine monophosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBR, peripheral benzodiazepine receptor; TSPO, translocator protein 18kDa.

* Corresponding author. Address: The Research Institute of the McGill University Health Center, Montreal General Hospital, 1650 Cedar Ave., Montreal, Quebec, Canada H3G 1A4. Tel.: +1 514 934 1934x44580; fax: +1 514 934 8439.
E-mail address: vassilios.papadopoulos@mcgill.ca (V. Papadopoulos).
[1] Present address: Department of Nutrition, American University of Technology, Halat, Lebanon.

0039-128X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.steroids.2012.08.019

mitochondrial membrane with its N-terminus facing the intermembrane space and its C-terminus facing the cytoplasm [8]. The C-terminus of TSPO contains a cholesterol recognition amino acid consensus (CRAC) motif which confers high affinity cholesterol binding to TSPO and plays a role in steroidogenesis [9–11]. To further our understanding of TSPO's cholesterol binding properties, we previously performed *in silico* and biochemical studies to identify alternative ligands for TSPO's CRAC motif. This work identified the androstenetriol 5-androsten-3β,17,19-triol (19-Atriol) as a ligand for TSPO and demonstrated that pharmacological inhibition of the TSPO CRAC motif was able to potently inhibit steroid production in steroidogenic cell models [12]. As structurally distinct androstenetriols have been identified to have diverse biological effects [13,14], we considered it of interest to examine the effects of a range of substituted steroids structurally homologous to 19-Atriol to better understand the structural nature of 19-Atriol's effect on steroidogenesis. In this study, we demonstrate that steroidogenic inhibitory activity of steroids structurally similar to 19-Atriol varied with the position of hydroxylation of the steroid nucleus, with the regions encompassing the 3, 7, 11, and 19 backbone carbons the most important for inhibitory activity. However, only the 19-substituted androstenetriol derivatives exhibited affinity for TSPO. Thus, we provide new insights into the structure–activity relationships of androstenetriols as modulators of steroidogenesis.

2. Experimental

2.1. Materials

The molecule [1,2,6,7-N-^{3}H]progesterone was obtained from Perkin Elmer (Waltham, MA). The modified cholesterol, 7-azi-5α-[3,5,6-^{3}H]cholestan-3β-ol ([^{3}H]azidocholesterol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Progesterone was obtained from Sigma–Aldrich (St. Louis, MO). Cell culture supplies were purchased from Life Technologies (Carlsbad, CA). Tissue culture plasticware was purchased from Corning (Corning, NY). All compounds used in this study were purchased from Steraloids, Inc. (Newport, RI), with the exception of 5-androsten-3β,17,19-triol (**1**), whose synthesis is described below. All other chemicals used were of analytical grade and were obtained from various commercial sources.

2.2. 5-androsten-3β,17,19-triol synthesis

Compound (**1**) was prepared by a chemical reduction of its corresponding ketone, 5-androsten-3β,19-diol-17-one (MW 304) (**2**), using the soft reagent sodium borohydride (Sigma–Aldrich, St. Louis, MO), which reduces aldehydes or ketones to the corresponding alcohols and may be used in aqueous or alcoholic solutions. The compound (**2**) (3.3 mmol) was dissolved in 7.5 volumes methanol (g/mL) and $NaBH_4$ was added slowly for a 1:2 M ratio with (**2**); the reaction was exothermic and the rate of addition was slow enough to not allow the reaction temperature to exceed 45 °C. After all the $NaBH_4$ had been added, the reaction was heated to a boil for 2 min, followed by rapid cooling on ice. The compound (**1**) was collected, washed twice with water and allowed to dry. Purity of (**1**) was determined by mass spectrometry.

2.3. In silico chemical clustering

Hierarchical structural clustering of the steroidal structures used in this study was performed using the ChemMine tools service [15].

2.4. Cell culture

MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (University of Iowa). Cells were cultured in 75 cm^2 cell culture flasks with Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 Ham supplemented with 5% fetal bovine serum and 2.5% heat-inactivated horse serum, as previously described [16].

2.5. Steroid biosynthesis

For steroid synthesis experiments in the presence of medium with dibutyryl-cAMP (dbcAMP) and 22*R*-hydroxycholesterol (22*R*-HC), MA-10 cells were plated onto 96-well plates at 5×10^4 - cells per well. After allowing cells to adhere for 3–18 h, the cells were washed with PBS and exposed to the respective treatment (1 mM dbcAMP or 20 μM 22*R*-HC in medium) in the presence or absence of the steroids under investigation. All media conditions were serum-free. The dbcAMP stocks were prepared in water. The 22*R*-HC and substituted steroid stocks were prepared as ethanolic stock solutions and used at a final ethanol concentration of <0.02% in media. Controls contained equivalent amounts of ethanol. At the end of the incubation, culture medium was collected and tested for progesterone production using radioimmunoassay (RIA) with progesterone antisera (MP Biomedicals, Solon, OH), following the manufacturer's recommended conditions. The sensitivity of the assay of the antibody was 15 pg/tube and the intra- and inter-assay coefficient of variation were 13% for each. Progesterone production was normalized for the amount of protein in each well and expressed as ng progesterone/mg protein. For presentation in figures, the percent change in progesterone production in the presence of drug relative to control values is shown. RIA data were analyzed using Prism 4.02 from GraphPad.

2.6. Mitochondrial integrity/cell viability

Cell viability and mitochondrial integrity at the end of the incubation protocols described above were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit for mitochondrial integrity [17] (Roche, Mannheim, Germany). Formazan blue formation was quantified at 600 nm and 690 nm using the Victor quantitative detection spectrophotometer (Perkin Elmer, Waltham, MA), and the results were expressed as (OD_{600}–OD_{690}).

2.7. Protein measurement

Proteins were quantified using the dye-binding assay of Bradford [18] with bovine serum albumin (BSA) as the standard.

2.8. [^{3}H]azidocholesterol photolabeling of synthetic CRAC peptide

[^{3}H]azidocholesterol photolabeling of synthetic CRAC peptides was performed as described previously for [^{3}H]promegestone with modifications [10]. Briefly, 100 μM synthetic TAT-CRAC peptide in phosphate-buffered saline (PBS) was incubated with [^{3}H]azidocholesterol at a final concentration of 100 nM in the absence or presence of 250 μM cholesterol (for non-specific binding) or steroids (**1**), (**2**), (**5**), (**8**), (**9**), or (**11**) in a 100 μL final volume. After a one h incubation at 4 °C, samples were photoirradiated for 30 min at a distance of 0.5 cm using UV light with a maximum emission at 366 nm (Ultraviolet Products, Gabriel, CA), and the assay was stopped by filtration through Whatman GF/C filters (Clifton, NJ) and washed with 10 mL of PBS. Bound radioactivity was determined by liquid scintillation counting and specific [^{3}H]azidocholesterol binding (taken as radioactive count in the presence of