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Investigating the anti-mineralocorticoid properties of synthetic progestins used in hormone therapy

Donita Africander [a], Renate Louw [a], Janet P. Hapgood [b,*]

[a] Department of Biochemistry, University of Stellenbosch, 7600 Stellenbosch, South Africa
[b] Department of Molecular and Cell Biology, University of Cape Town, Private Bag, Rondebosch 7700, South Africa

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ABSTRACT

A more detailed understanding of the affinities and efficacies for transcriptional regulation by the synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) via the mineralocorticoid receptor (MR) is required, to better understand their relative risk profiles. Both MPA and NET-A bind to the MR, although with about 100-fold lower affinities than that of Prog. MPA and NET-A exhibit no agonist activity, but NET-A, unlike MPA, has similar antagonistic efficacy to Prog on the endogenous mineralocorticoid/glucocorticoid response element (MRE/GRE)-containing genes, α-glycolytic protein or orosomucoid-1 (Orm-1) and plasminogen activator inhibitor-1 (PAI-1). This study is the first to show that NET-A, but not MPA, can dissociate between transrepression and transactivation via the MR. Given the relatively low affinity and potency of MPA and NET-A for the MR, our results suggest that these progestins are unlikeley to exert significant effects via the MR at doses used in hormonal therapy. However, considering their relative free concentrations compared to endogenous hormones, the possibility that NET-A may exhibit significant MR antagonist activity, with some possible cardiovascular protective benefits, should not be excluded.

1. Introduction

Synthetic progestins have been used for decades for contraception and hormone replacement therapy (HRT). However, concerns have been raised regarding side-effects on breast cancer, cardiovascular complications and immune function and susceptibility to infections (reviewed in [1–5]); [6–9]. While a wide range of different progestins are currently available, particular concern has been raised regarding the use of MPA in both HRT and contraception [7,5]. NET-A and its derivatives are widely used in HRT, while MPA and NET-A are the two most commonly used injectable contraceptives particularly in the developing world. Many clinical studies have thus focused on these two progestins. MPA and NET used in HRT may increase the incidence of breast cancer [6,8], and MPA may have adverse effects on cardiovascular health [9]. Furthermore, MPA but not NET is implicated in increasing risks of HIV-1 infectivity and transmission [2]. As progestins are designed to mimic the actions of the endogenous hormone progesterone (Prog), their biological effects are assumed to be mediated by the progesterone receptor (PR). However, progestins exhibit a wide range of activities, most likely reflecting their differential affinities and activities via the mineralocorticoid- (MR), glucocorticoid- (GR) and androgen-receptors [1,3,4], thus raising the possibility that their side-effects, and some of the beneficial effects may be mediated by steroid receptors other than the PR. Relatively little research has been conducted to investigate detailed molecular mechanisms of progestins via different steroid receptors. Here we focus on the mechanism of action of MPA and NET-A via the MR, with a view to better understand and predict side-effects on cardiovascular and other physiological functions.

The MR is expressed in several tissues such as the kidney, heart, central nervous system, and the vasculature [1,4] and thus may play a role in responses to progestins targetting the MR. MR-mediated increase in transcription of target genes is termed transactivation, and occurs when ligand-activated MR binds to MREs in the promoters of specific genes [1,4]. Ligand-activated steroid receptors can also negatively regulate genes (transrepression), most likely via protein–protein interactions between the receptor and other transcription factors such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1) [1,10].

Abbreviations: Prog, progesterone; MR, mineralocorticoid receptor; HRT, hormone replacement therapy; MPA, medroxyprogesterone acetate; NET, norethisterone; NET-A, norethisterone acetate; NET-EN, norethisterone enanthate; Ald, aldosterone; MRE/GRE, mineralocorticoid/glucocorticoid response element; GR, glucocorticoid receptor; PR, progesterone receptor; Orm-1, α-glycolytic protein or orosomucoid-1; PAI-1, plasminogen activator protein-1; AP-1, activator protein-1.

* Corresponding author. Address: Department of Molecular and Cell Biology, University of Cape Town, Private Bag, Rondebosch 7700, South Africa. Fax: +27 21 6897573.
E-mail address: Janet.Hapgood@uct.ac.za (J.P. Hapgood).

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Although contentious [11], there is some evidence that inappropriate activation of the MR by aldosterone (Ald), an endogenous mineralocorticoid, leads to deleterious effects on the cardiovascular system, while intervention with a MR antagonist is beneficial to patients with heart failure (reviewed in [12]). Ald has been shown to regulate expression of genes that may contribute to the development of cardiovascular damage such as Orm-1, an inflammation/acute phase-related gene, and PAI-1, an antithrombolytic factor that promotes tissue fibrosis [13], suggesting a beneficial mechanism for MR antagonists. Since progestins such as drospirenone and trimegestone were developed to have anti-mineralocorticoid properties to exhibit beneficial effects on blood pressure and cardiovascular function [1], ligands such as MPA and NET-A that reportedly lack anti-mineralocorticoid activity, may lead to cardiovascular complications. The mechanism of action and target genes, of MPA and NET-A, as compared to Prog, via the MR. remains poorly defined. Thus, the present study aimed to provide a comparative biochemical profile of the actions of these ligands via the human MR (hMR), in the same model system.

2. Materials and methods

2.1. Plasmids

pTAT-GRE-E1b-luc, driven by the E1b promoter containing two copies of the rat TAT-GRE, has been described previously [14]. The cytomegalovirus (CMV)-driven-β-galactosidase expression vector (pCMV-β-gal), the 7xAP-1-luc plasmid, the pRShMR [15] and the pRS-hGR α plasmids expressing the human MR and GR, respectively, were obtained from Guy Haegeman (University of Gent, Belgium), Stratagene (Houston, Texas, USA) and Prof. Evans (Howard Hughes Medical Institute, La Jolla, USA), respectively. FuGENE6 (Roche Molecular Biochemicals, South Africa) was used for all transfections, according to the manufacturer's instructions.

2.2. Inducing compounds

11β,21-Dihydroxy-3,20-dioxo-4-pregnen-18-al (Ald), 7α-acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone (spironolactone), 4-pregnene-3, 20-dione (Prog), 6α-methyl-17α-hydroxyprogesterone acetate (MPA), 17α-ethynyl-19-nortestosterone 17β-acetate (NET-A) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma–Aldrich, South Africa. All test compounds were dissolved in absolute ethanol and added to serum-free culturing medium in a final concentration of 0.1% ethanol. Vehicle control incubations contained 0.1% ethanol.

2.3. Whole cell binding assays

COS-1 monkey kidney cells were maintained as previously described [16]. Competitive whole cell binding assays were performed and analyzed essentially as described by Bamberger et al. (1995) [17], with the following modifications [16]. COS-1 cells were seeded into 24-well tissue culture plates at 1×10^5 cells per well. On day 2, cells were transfected with 0.375 μg pRShMR expression vector and 0.0375 μg of the pCMV-β-gal expression vector. On day 3, the cells were washed with phosphate-buffered saline (PBS) prior to incubation with 0.2 nM [^{3}H]-Ald (87.9 Ci/mmol) (PerkinElmer Life and Analytical Science, South Africa), in the absence or presence of increasing concentrations of unlabelled Ald, Prog, MPA or NET-A in serum-free DMEM for 16 h at 37 °C. Total binding ([^{3}H]-Ald only) was determined by scintillation counting and expressed as 100%. Specific bound [^{3}H]-Ald was calculated as the difference between total and non-specific binding ([^{3}H]-Ald plus 10 μM unlabelled Ald) and expressed as a relative% of total binding. K_i values for Prog, MPA and NET-A were determined from the heterologous displacement curves using the EC_{50}s, K_d for Ald, and concentration of [^{3}H]-Ald, as previously described [18].

2.4. Luciferase reporter assays

For transactivation: COS-1 cells were seeded into 96-well tissue culture plates at 1×10^4 cells per well. On day 2, cells were transiently transfected with 50 ng pTAT-GRE-E1b-luc, 5 ng pRShMR, and pCMV-β-gal expression vectors, respectively. On day 3, the cells were washed with PBS and incubated with 1 μM of either Ald, Prog, MPA or NET-A in serum-free DMEM for 24 h to investigate agonist activity. For antagonist activity, cells were incubated with 1 nM Ald in the absence and presence of increasing concentrations of Prog, MPA or NET-A.

For transrepression: COS-1 cells were seeded into 24-well tissue culture plates at a density of 5×10^4 cells per well. On day 2, cells were transfected with 0.045 μg pRShMR, 0.09 μg 7xAP-1-luc and 0.0225 μg pCMV-β-Gal expression vectors, respectively. On day 3, the cells were washed with PBS and incubated with serum-free medium containing 10 ng/ml PMA and 1 μM of each test compound in serum-free DMEM for 24 h. For both assays, cells were lysed and analyzed as previously described [19].

2.5. Isolation of total RNA and real-time quantitative RT-PCR analysis

H9C2 rat cardiomyocyte cell line were maintained as previously described for COS-1 cells [16]. Cells were seeded into 12-well plates at a density of 1×10^5 cells/well, transiently transfected with 70 ng pRShMR, and grown in serum-free DMEM for 48 h. Cells were incubated with 1 nM Ald in the absence and presence of 1 μM Prog, MPA, NET-A or Spironolactone for 24 h. Total RNA was isolated, cDNA prepared and realtime PCR performed as previously described [19]. The thermal cycling parameters were: initial denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 57 °C for 30 s, using primers previously described (Supplementary Table 1) [13,20].

2.6. Data manipulation and statistical analysis

The Graph Pad Prism® software was used for data analysis. Non-linear regression and one site competition were used in whole cell binding assays, whereas non-linear regression and sigmoidal dose response were used in transactivation (antagonist) experiments. One-way ANOVA analysis of variance and Newman-Keuls or Bonferroni (compares all pairs of columns) posttests were used for statistical analysis. Statistical significance of differences is indicated by *, ** or ***, to indicate $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively, whereas no statistical significance is indicated by $p > 0.05$. The letters a, b, c etc. are also used to denote statistically significant differences, where all those values which differ significantly from others, are assigned a different letter.

3. Results

3.1. MPA and NET-A have a similar binding affinity for the MR

Competitive whole cell binding assays were performed in the steroid-receptor-deficient COS-1 cell line expressing exogenous hMR. To obtain accurate K_d and K_i values an appropriate concentration of [^{3}H]-Ald, in the range two to ten times lower than the EC_{50}, was established as 0.2 nM, and the incubation time required for equilibrium to be reached for 0.2 nM [^{3}H]-Ald binding to the MR, was determined as sixteen hours (Supplementary Fig. 1). Homologous/heterologous curves with unlabelled steroids were then