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Effect of retinoic acid on human adrenal corticosteroid synthesis

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ABSTRACT

Aims: Retinoic acid has recently yielded promising results in the treatment of Cushing's disease, *i.e.*, excess cortisol secretion due to a pituitary corticotropin (ACTH)-secreting adenoma. In addition to its effect on the tumoral corticotrope cell, clinical results suggest an additional adrenal site of action. Aim of this study was to evaluate whether retinoic acid modulates cortisol synthesis and secretion by human adrenals *in vitro*.
Main methods: Primary cultures from 10 human adrenals specimens were incubated with 10 nM, 100 nM and 1 μM retinoic acid with and without 10 nM ACTH for 24 h. Cortisol levels were measured by radioimmunoassay and *CYP11A1*, *STAR* and *MC2R* gene expression analyzed by real-time PCR.
Key findings: Retinoic acid increased cortisol secretion (149.5 ± 33.01%, 151.3 ± 49.45% and 129.3 ± 8.32% control secretion for 10 nM, 100 nM and 1 μM respectively, $p < 0.05$) and potentiated *STAR* expression (1.51 ± 0.22, 1.56 ± 0.15 and 1.59 ± 0.14 fold change over baseline, for 10 nM, 100 nM and 1 μM respectively, $p < 0.05$). Concurrently, retinoic acid markedly blunted constitutional and ACTH-induced *MC2R* expression (0.66 ± 0.11, 0.62 ± 0.08 and 0.53 ± 0.07 fold change over baseline, for 10 nM, 100 nM and 1 μM respectively, $p < 0.05$; 0.71 ± 0.10, 0.51 ± 0.07 and 0.51 ± 0.08 fold change over ACTH alone, for 10 nM, 100 nM and 1 μM respectively, $p < 0.05$). No effect on *CYP11A1* was observed.
Significance: Retinoic acid stimulates cortisol synthesis and secretion in human adrenals and at the same time markedly blunts ACTH receptor transcription. These results reveal a novel, adrenal effect of retinoic acid which may contribute to its efficacy in patients with Cushing's disease.

1. Introduction

Retinoic acid is produced by the adrenal gland [1] and is involved in adrenal embryonic development [2]. *In vitro* studies have also shown that retinoic acid modulates corticosteroid secretion and cell proliferation in adrenal cancer cell lines [3,4]. Most recently, retinoic acid has been proposed for the treatment of Cushing's disease, *i.e.*, excess cortisol secretion due to a pituitary corticotropin (ACTH)-secreting tumor, a severe endocrine disorder lacking efficacious medical therapy. In fact, retinoic acid was shown to reduce ACTH secretion and inhibit cell proliferation in a murine corticotrope tumor cell line [3,5,6]. Our pilot study in patients with Cushing's disease [7] showed that administration of retinoic acid reduced and normalized excess cortisol secretion in a good proportion of patients. During the course of this study we observed that retinoic acid treatment resulted in a more potent decrease in cortisol than in ACTH levels. While this effect may be due to the interplay between the two hormones [8], it could also be due to a direct, adrenal action.

In Cushing's disease, the adrenal gland is either normal or hyperplastic due to continued stimulation by ACTH [9]. Thus, evidence collected in adrenal cancer [3,4] may apply only in part. It appeared therefore of interest to evaluate the effect of retinoic acid on the normal human adrenal cortex, in particular as regards cortisol secretion and ACTH-activated steroidogenic gene expression.

2. Experimental

2.1. Adrenal cultures

Primary cultures from 10 human adrenal specimens were studied. Normal human adrenal tissue was obtained from subjects submitted to adrenalectomy for aldosteronism or non-functioning adenomas. None of the patients presented clinical or hormonal features of hypercortisolism. Adrenals cultures were established as per our usual protocols [10,11]. In brief, adrenals were minced, digested in 0.1% collagenase, plated at approx. 300,000 cells/well, incubated in DMEM supplemented with 10% fetal bovine serum and antibiotics for 3–5 days to

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allow attachment. The study was approved by our Institution's Ethical Committee and informed consent obtained from patients prior to surgery by the referring surgeon.

2.2. Treatments and assays

Cells were washed in DMEM containing 0.1% BSA for 1 h prior to incubation with 10 nM, 100 nM and 1 μM 9-cis retinoic acid (Sigma Aldrich, St. Louis, USA) alone and together with 10 nM ACTH for 24 h. Pregnenolone (10 μM) was included in the test medium in order to promote steroidogenesis [12]. Both pregnenolone and retinoic acid were dissolved in 100% ethanol and diluted 1000-fold in DMEM; equal volumes of ethanol were added to control wells. In each adrenal, treatments were performed in triplicate. Cortisol was measured in medium using Coat-A-Count radioimmunoassay (Siemens Healthcare Diagnostics, Erlangen, Germany) and normalized to control or ACTH-stimulated levels, respectively.

2.3. Quantitative real-time PCR

RNA was extracted from plated cells using TRIzol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's instruction. The amount and the quality of the RNA were checked on nanophotometer (Implen GmbH, München, Germany) and 100 ng RNA reverse-transcribed with Superscript Vilo cDNA Synthesis Kit (Life Technologies, Carlsbad, USA). Quantitative Real-Time PCR was performed on a 7900 HT sequence Detection System (Applied Biosystems, Foster City, USA), using the Platinum Quantitative PCR Supermix-UDG with ROX (Life Technologies, Carlsbad, USA). The following genes were evaluated: *CYP11A1* (Taqman probe Hs00167984_m1), *STAR* (Hs00264912_m1), *MC2R* (Hs00300820_s1) and normalized to *RPLP0* (Hs99999902_m1). Expression data are analyzed as $2^{-\Delta\Delta Ct}$ and expressed as fold increase.

2.4. Statistical analyses

Data are expressed as mean ± standard error of the mean (S.E.M.) and relative to control or ACTH-stimulated wells for each adrenal specimen. Comparisons were performed using Wilcoxon's signed-rank test and statistical significance accepted at $p < 0.05$.

3. Results

Retinoic acid increased spontaneous cortisol secretion by human adrenals at all concentrations tested: 10 nM: 149.5 ± 33.01%, 100 nM: 151.3 ± 49.45% and 1 μM: 129.3 ± 8.32% unstimulated secretion (all $p < 0.05$ *vs* control; Fig. 1, left panel).

As regards expression of steroidogenic genes, retinoic acid induced a substantial increase in steroidogenic acute regulatory protein (*STAR*) gene expression (10 nM: 1.51 ± 0.22, 100 nM: 1.56 ± 0.15 and 1 μM: 1.59 ± 0.14 fold change over control, all $p < 0.05$ *vs* control; Fig. 2, right panel) and reduced expression of the ACTH receptor, melanocortin type receptor (*MC2R*; 10 nM: 0.66 ± 0.11, 100 nM: 0.62 ± 0.08 and 1 μM: 0.53 ± 0.07 fold change over control, all $p < 0.05$ *vs* control; Fig. 2, left panel) compared to unchallenged wells. No significant changes in *CYP11A1*, the gene coding for cytochrome P450 cholesterol side-chain cleavage, the first step in adrenal steroidogenesis, were observed during treatment with retinoic acid (10 nM: 1.11 ± 0.11, 100 nM: 1.02 ± 0.05 and 1 μM: 1.03 ± 0.05 fold change over control, all N.S.)

Incubation with 10 nM ACTH evoked the expected increase in cortisol secretion (208.9 ± 63.71% constitutive secretion, $p < 0.01$) and *CYP11A1* (2.11 ± 0.28 fold change over control wells, $p < 0.05$), *STAR* (3.73 ± 1.08 fold change over control wells, $p < 0.05$) and *MCR2* gene expression (11.9 ± 3.21 fold change over control wells, $p < 0.01$). Co-incubation with retinoic acid induced a noticeable though non-significant increase in ACTH-stimulated cortisol secretion (10 nM: 139.8 ± 21.69%, 100 nM: 147.4 ± 25.48% and 1 μM: 131.7 ± 24.48% ACTH-stimulated secretion, N.S.; Fig. 1, right panel). Of note, the increase induced by retinoic acid during ACTH co-incubation did not differ from the increase observed in absence of ACTH (paired comparison at all three retinoic acid concentrations N.S.).

Retinoic acid co-incubation blunted the increase in *MC2R* expression induced by ACTH (10 nM: 0.71 ± 0.10 fold change, 100 nM: 0.51 ± 0.08 fold change and 1 μM 0.52 ± 0.08 fold change over ACTH-stimulated wells, all $p < 0.05$) whereas no significant changes were observed as regards *CYP11A1* (10 nM: 1.19 ± 0.10 fold change, 100 nM 1.15 ± 0.13 fold change and 1 μM 1.07 ± 0.12 fold change over ACTH-stimulated wells, all N.S.) and *STAR* (10 nM: 1.07 ± 0.19 fold change, 100 nM: 1.07 ± 0.22 fold change and 1 μM 1.37 ± 0.23 fold change over ACTH-stimulated wells, all N.S., Supplemental Fig. 1). Changes in *MCR2* induced by retinoic acid with or without ACTH were comparable for all three retinoic acid concentrations (all comparisons N.S.).

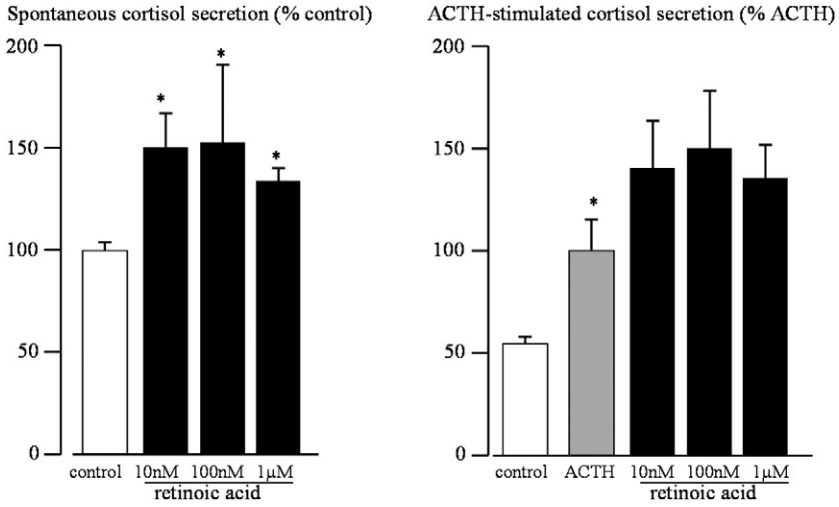

Fig. 1. Mean cortisol changes in 10 human adrenal tissue primary cultures after 24 h incubation with retinoic acid. Left panel shows spontaneous secretion (control wells = 100%), right panel shows ACTH-stimulated concentrations (ACTH stimulated wells = 100%). White bar: control wells, graybar: incubation with 10 nM ACTH; black bars: incubation with 10 nM, 100 nM or 1 μM retinoic acid alone (left panel) or with 10 nM ACTH (right panel). * denotes $p < 0.05$ significance *vs* control.

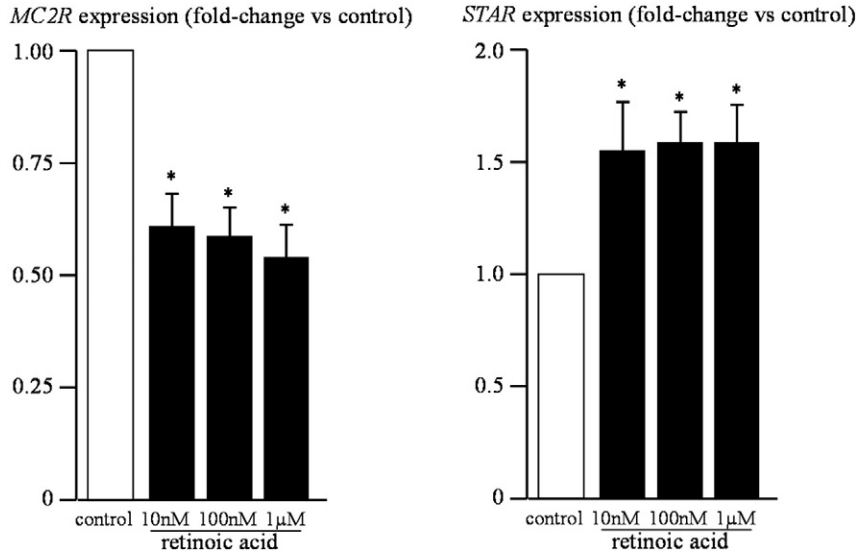

Fig. 2. *MC2R* and *STAR* expression in human adrenal primary cultures after 24 h incubation with retinoic acid. Left panel shows mean *MC2R* fold changes *vs* control wells, right panel shows mean *STAR* fold changes *vs* control wells. White bar: control wells, black bars: incubation with 10 nM, 100 nM or 1 μM retinoic acid. * denotes $p < 0.05$ significance *vs* control.

4. Discussion

The role of retinoic acid in adrenal embryonic development is well known [2], indeed, retinoic acid-mediated transcription has been hypothesized to play a role in adrenal hypoplasia due to *DAX-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1) mutations [13,14]. In adrenal cancer cell lines, retinoic acid has been shown to modulate corticosteroid secretion [3,4], thus suggesting a role also later in life.

In parallel to this evidence, retinoic acid has recently been proposed as a potential targeted therapy for Cushing's disease due to its action on the tumoral corticotrope. Indeed, studies using a corticotrope tumor cell line, AtT-20, reported that retinoic acid inhibits proopiomelanocortin (*POMC*) synthesis and ACTH release via inhibition of Nur77/Nurr1 and AP-1 (activator protein-1) [3,6]. Further, retinoic acid exerted an anti-proliferative effect on AtT-20 cells which appeared to rely on induction of bone morphogenetic protein-4 (BMP-4) [5,15], a member of the transforming growth factor β (TGF-β) family with known actions on pituitary tumorigenesis [16] and of caspase 3 and 8 [6]. In this cell line, retinoic acid also increased sensitivity to dopaminergic agents both as regards cell viability and inhibition of *POMC* and ACTH [17]. An inhibitory effect of retinoic acid was also observed in human tumoral corticotrope primary cultures [3,17] which led to a pilot clinical trial in patients with Cushing's disease [7]. Result of this study proved promising as did a study in dogs with endogenous hypercortisolism [18], thereby confirming the potential role of retinoic acid in treatment of Cushing's disease.

These latter studies also suggested an additional action downstream to the pituitary, *i.e.*, on the adrenal, given that the effect of retinoic acid on cortisol levels was more marked than the change in plasma ACTH [7]. While this phenomenon is not uncommon for treatments which target the pituitary in Cushing's disease [7,8,19], it could also be due to a direct adrenal action. Given all the above, we decided to investigate the effect of retinoic acid on cortisol secretion and on the synthesis of genes involved in ACTH-stimulated steroid biosynthesis.

Our results show that retinoic acid stimulates cortisol release by normal human adrenal tissue and further, modulates *STAR* and *MC2R*, two key factors in ACTH-driven steroidogenesis. Spontaneous cortisol release was increased on average by 30–50% during retinoic acid incubation whereas enhancement of the ACTH-stimulated cortisol response was less evident, possibly a consequence of its action on *MC2R* (see below).

Retinoic acid increased the expression of *STAR*, the rate-limiting enzyme for adrenal cholesterol availability, thus triggering steroidogenesis and rapid cortisol secretion [20]. Increased adrenal *STAR* synthesis and protein expression has also been observed during liver X receptor (LXR) agonist treatment in mice and appears to be due to LXR-RXR (retinoid X receptor) heterodimer binding to the *STAR* promoter [21]. Similar results have also been observed in mouse Leydig cells treated with retinoic acid [22,23]. Conversely, no significant changes in expression of gene coding for the first step in adrenal steroidogenesis, *i.e.*, P450 cholesterol side-chain cleavage, were observed on retinoic acid, both with and without ACTH co-incubation. *CYP11A1* expression increased as expected during incubation with ACTH alone [20] but does not seem to be a direct target of retinoic acid signaling in the normal adrenal gland.

Concurrently with increased cortisol release and *STAR* expression, retinoic acid markedly reduced ACTH receptor expression. This was evident for baseline, constitutional *MC2R* synthesis as well as during stimulation with ACTH. In fact, the customary ACTH-induced increase in *MC2R* [24,25] was nearly halved. This indicates that retinoic acid interferes with mechanisms involved in adrenal ACTH receptor expression, *e.g.*, steroidogenic factor 1 (SF-1), cAMP, DAX-1 [26], or acts directly upon the *MC2R* promoter. On the other hand, long-term treatment of mice with an LXR agonist was associated with increased adrenal *MC2R* expression [27], possibly a corollary to the known activation of the hypothalamo-pituitary-adrenal axis by retinoids *in vivo* [28,29].

Perhaps not surprisingly, our data is in contrast with results obtained in adrenal cancer cell lines [3,4]. Studies performed on mouse and human adrenal cancer cell lines reported an inhibitory effect of retinoic acid on spontaneous and forskolin-stimulated steroid secretion [3,4]. Reduced expression of several enzymes involved in steroid hormone biosynthesis, *e.g.*, *CYP11A1*, *HSD3B2*, *STAR*, had also been observed during retinoic acid or LXR/RXR agonist treatment in adrenal cancer cell lines [4,30]. On the other hand, retinoic acid signaling and production is altered in adrenal cancer [31,32] and, further, tumoral adrenal tissue lacks COUP-TF I/II (chicken ovalbumin upstream promoter transcription factor I/II), one of the nuclear receptors involved in retinoic acid signaling [33], which, conversely, is expressed in normal adrenal cells [34,35]. In Cushing's disease, adrenals are either normal or hyperplastic [9] thus effects observed in neoplastic adrenals are unlikely to apply. Given our

results on normal adrenals, further experiments on hyperplastic adrenal tissues from patients with Cushing's disease are certainly worth performing.

5. Conclusions

Our findings indicate that retinoic acid exerts a stimulatory effect on cortisol synthesis and release by the normal adrenal. Concurrently, retinoic acid markedly reduces expression of the adrenal ACTH receptor. These findings suggest that the effect of retinoic acid in Cushing's disease is the result of the interplay between its action at pituitary and at adrenal level.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2016.03.023.

Conflict of interest

The authors declare that there are no conflicts of interest.

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None.

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