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Journal of Steroid Biochemistry & Molecular Biology 92 (2004) 469–476
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Dehydroepiandrosterone and its 7-hydroxylated metabolites do not interfere with the transactivation and cellular trafficking of the glucocorticoid receptor

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Received 21 June 2004; accepted 8 October 2004

Abstract

The human brain is a target tissue for glucocorticoids (GC). Dehydroepiandrosterone (DHEA) is a neurosteroid produced in the brain where it is transformed into 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. The antiglucocorticoid effects of both 7-hydroxylated metabolites have been investigated with evidence in mice that neither form of DHEA interfered with the binding of GC to its glucocorticoid receptor (GR), but contributed to a decreased nuclear uptake of the activated GR. Our objective was to use COS-7 cell culture to research DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA interferences with GR trafficking. These cells did not carry out the 7α-hydroxylation of DHEA and the oxidation of cortisol into cortisone. The cDNA of the human GR was inserted into pcDNA3 for a transient transfection of COS-7 cells. Human GR transactivation activity was measured from a luciferase-MMTV reporter gene. The transfected COS-7 cells were cultured using 10^{-12} to 10^{-5} M dexamethasone (DEX) or cortisol, which triggered the reporter expression. Treatment with 10^{-12} to 10^{-5} M DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA caused no change in the GC-induced GR transactivation. A reconstruction of the process associated EGFP to the human GR cDNA. Confocal microscopic examination of COS-7 cells transiently expressing the fusion protein EGFP-GR showed nuclear fluorescence 60 min after incubation with 10^{-8} M DEX or cortisol. The addition of 10^{-5} M DHEA, 7α-hydroxy-DHEA or 7β-hydroxy-DHEA did not change its kinesis and intensity. These results contribute to the knowledge of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA, in relation to antiglucocorticoid activity. We conclude that direct interference with GR trafficking can be discounted in the case of these hormones, therefore proposing new possibilities of investigation.
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Keywords: 7α-Hydroxy-DHEA; 7β-Hydroxy-DHEA; Cortisol; Dexamethasone; COS-7 cells; Transient transfection

1. Introduction

Antiglucocorticoid activity has been considered an effect in response to dehydroepiandrosterone (DHEA) administered to animals [1]. This result could not be obtained after in vitro works, thus the possibility that DHEA metabolites could be the active molecular forms was suggested [1]. Investigation of DHEA metabolism had shown that a 7α-hydroxylated metabolite was produced by a cytochrome P450 termed P4507B1 [2]. This catalyzation has been demonstrated in the rat [3,4], mouse [5,6] and human species [7] with reports of age-related level changes in plasma [8]. Aside from 7α-hydroxy-DHEA, a production of 7β-hydroxy-DHEA has been discovered and measured in rats [3], mice [9–11] and humans [12,13]. The 7β-hydroxy-DHEA production required an active P4507B1 [14] and could have derived from 7α-hydroxy-DHEA [15]. Both 7-hydroxylated-DHEA metabolites have been shown to increase the immune response in mice and humans [9,16], with a greater potency for the 7α-epimer. Further research with radio-labelled dexamethasone (DEX) of the putative antiglucocorticoid effect in mouse

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doi:10.1016/j.jsbmb.2004.10.014

cells from the liver, brain, thymus and spleen had indicated that 7-hydroxylated DHEA metabolites did not interfere with the binding of DEX to the glucocorticoid receptor (GR) but there was some interference with the activated GR trafficking [17]. This evidence required confirmation through different approaches to human GR (hGR) cell-trafficking. Consequently, COS-7 cells were selected for the study because of their lack of 7α-hydroxylation capacity shown in preliminary experiments and reported use in GR transient transfection assays [18,19]. Results with the hGR construct transiently expressed in the COS-7 cells indicated that the 7-hydroxy-DHEA antiglucocorticoid effect resulted without impairment to hGR cellular trafficking.

2. Materials and methods

2.1. Chemicals

Cortisol, cortisone, DEX and DHEA were obtained from Sigma–Aldrich (L'Isle d'Abeau Chesnes, France). RU486 was a gift from Aventis (Romainville, France). Custom chemical synthesis by Roowin SA (Paris, France) provided milligram quantities of chemically pure 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. [4-^{14}C]-Cortisol (53.5 mCi/mmol) and [4-^{14}C]-DHEA (47.8 mCi/mmol) were purchased from Perkin–Elmer Life Sciences, Inc. (Boston, MA, USA). [4-^{14}C]-7α-Hydroxy-DHEA (47.8 mCi/mmol) was produced as previously described [12]. All products for the biochemical studies were from Sigma–Aldrich. Dulbecco's minimal essential medium (DMEM) and all other compounds for cell culture were from Invitrogen (Cergy Pontoise, France).

2.2. Expression and reporter constructs

The expression plasmid pchGR contains the entire hGR coding sequence [20]. The pEGFP-GR was constructed as follows. The pchGR was digested with the *Bpu*10I restriction enzyme and ligated using the forward oligonucleotides linkers 5′ TCATTCCGGAAT 3′ and 5′ TGAATTCCGGAA 3′, introducing a *Bsp*EI restriction site and leading to a 20 amino acids sequence (MDSKESLTPGREENPSSVLA) deletion at the N-terminal end. The $hGR_{21–777}$ was excised from this construct by *Bsp*EI and *Xho*I and inserted into the pEGFP-C1 vector (BD Biosciences Clontech, Ozyme, St Quentin en Yvelines, France). The plasmid pcβgal was constructed by cutting out the *Hin*dIII–*Bam*HI fragment coding for the β-galactosidase enzyme from the plasmid pSVβ (Promega, Charbonnières, France) and inserting it into the pcDNA3 (Invitrogen, Cergy Pontoise, France). The pFC31Luc contains the mouse mammary tumor virus (MMTV) promoter that actuates the luciferase gene [21].

2.3. Cultured cells and transfection procedures

COS-7 cells were cultured in T175 flasks of DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were maintained as above in 10% charcoal-stripped FCS conditions for 4 h and then used throughout the transfection procedure. The charcoal-stripped FCS was prepared from FCS that was stripped of steroids by two extractions with dextran-coated charcoal. Cells at subconfluence were transfected by the phosphate calcium precipitation method. The phosphate solution, prepared for one T175 flask, contained 15 μg of one of the receptor expression vectors (pchGR or pEGFP-GR), 30 μg pFC31Luc and 6 μg pcβgal in HEPES-buffered saline 1X supplemented with 160 mM $CaCl_2$.

2.4. Receptor transactivation activity

Twelve hours after transfection the cells were rinsed with phosphate-buffered saline, trypsinized, and transferred in six-well plates at a density of 10^5 cells per well. The steroids to be tested were added to the cells 4 h after their seeding and were incubated with transfected cells. After 24 h of incubation, cell extracts were assayed for luciferase [22] and β-galactosidase activities [23]. To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by optical density obtained in the β-galactosidase assay.

2.5. Confocal microscopy

The COS-7 cells were transfected with pEGFP-GR two days before microscopy. They were seeded at a density of 8×10^4 cells in a self-enclosed chambered coverglass (Lab-Tek, Nalge Nunc International, Naperville, IL) treated with a 2 mg/ml rat tail collagen type 1 solution in 0.1% acetic acid (Institut Jacques Boy, Reims, France) that was diluted 50-fold in 60% ethanol. The cells were kept at 37 °C in the presence of 5% CO_2 and imaged on a laser scanning confocal microscope (LSM510, Zeiss, Oberkochen, Germany). The EGFP was excited with the 488 nm line from an argon laser.

2.6. Steroid metabolism

Intact COS-7 cells were seeded in six-well plates at a density of 10^5 cells per well. Unlabelled cortisol, DHEA, or 7α-hydroxy-DHEA (10^{-6} M) was added to the cells together with 50,000 dpm of the corresponding radio-labelled steroid ([4-^{14}C]-cortisol, [4-^{14}C]-DHEA, or [4-^{14}C]-7α-hydroxy-DHEA). After 24 h of incubation in DMEM supplemented with 10% charcoal-stripped FCS, the reactions were stopped by the addition of 0.5 ml acetone. The metabolites were extracted five times with 2 ml ethyl acetate from the culture medium and the organic phases were pooled to evaporate. The extracts were then dissolved in 150 μl of ethyl acetate and applied to silica 60-coated glass plates (Merck, Darmstadt, Germany) before separation by thin-layer chromatography (TLC) with migration in ethyl acetate for DHEA and 7α-hydroxy-DHEA metabolism and $CHCl_3$/ethanol/H_2O

(87:13:1, v/v) for cortisol metabolism. Auto-radiography of thin-layer chromatograms with BioMax-light X-ray film (Kodak) revealed the substrates and their metabolites; they were identified by comparing their Rf with reference steroids. Quantitative scanning of the plates was carried out with the use of a Berthold automatic TLC-linear analyser (Perkin–Elmer, France).

3. Results

3.1. DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA do not interfere with the hGR transactivation activity in COS-7 cells

The COS-7 cells were transiently transfected with pchGR and pFC31Luc as a glucocorticoid (GC) response element-containing reporter plasmid, and pcβgal as an internal control for transfection efficiency. Each steroid (10^{-12} to 10^{-5} M concentrations of DEX, cortisol, RU486, cortisone, DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA) was tested for agonistic activity during 24 h before harvesting the transiently transfected COS-7 cells. As expected, DEX and cortisol increased the hGR transactivation activity in a dose-dependent manner through expression of luciferase activity, but the RU486 and cortisone levels did not change it. The ED_{50} of DEX and cortisol (10^{-9} and 10^{-8} M, respectively) differed by one order of magnitude. There was no evidence for agonistic activity according to tests carried out with DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA (Fig. 1).

This experimental model was also used to test the putative antagonistic activities of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA towards GC-induced transactivation activity of the hGR. That is, transiently transfected COS-7 cells were incubated with increasing concentrations of the steroids (10^{-12} to 10^{-5} M) for one hour, after which 10^{-7} M DEX or 10^{-6} M cortisol was added for 24 h processing. As expected, RU486 antagonized the transactivation activity of the hGR in a dose-dependent manner (IC_{50} 5×10^{-8} M) (Fig. 2). These tests as described above provided no evidence for antagonistic activity. Cortisone at 10^{-6} and 10^{-5} M inhibited the DEX-induced hGR transactivation activity by 20% (Fig. 2A). When cortisol was used in the place of DEX (Fig. 2B), RU486 antagonized the transactivation activity of the hGR in a dose-dependent manner (IC_{50} 5×10^{-8} M), while cortisone, DHEA and its 7-hydroxylated metabolites showed no antagonistic activity.

3.2. DHEA and 7-hydroxy-DHEA do not interfere with the activated hGR trafficking in COS-7 cells

Preliminary experiments with the pEGFP-GR construction showed that the transiently transfected COS-7 cells responded to DEX with an ED_{50} of 5×10^{-8} M (data not shown). Typical fluorescence images of the living cells were captured every 5 min with confocal microscopy. Untreated cells showed the hGR-associated fluorescence in the cytoplasm only (t_0 in Figs. 3–5). The use of 10^{-8} M cortisol or 10^{-8} M DEX for 60 min triggered a complete transfer of the EGFP fluorescence from the cytoplasm to the nuclei (Fig. 3). The use of 10^{-5} M DHEA, 7α-hydroxy-DHEA or 7β-hydroxy-DHEA in the cell medium did not modify the cytoplasmic localization of the hGR as shown by the maintenance of EGFP fluorescent intensity in the

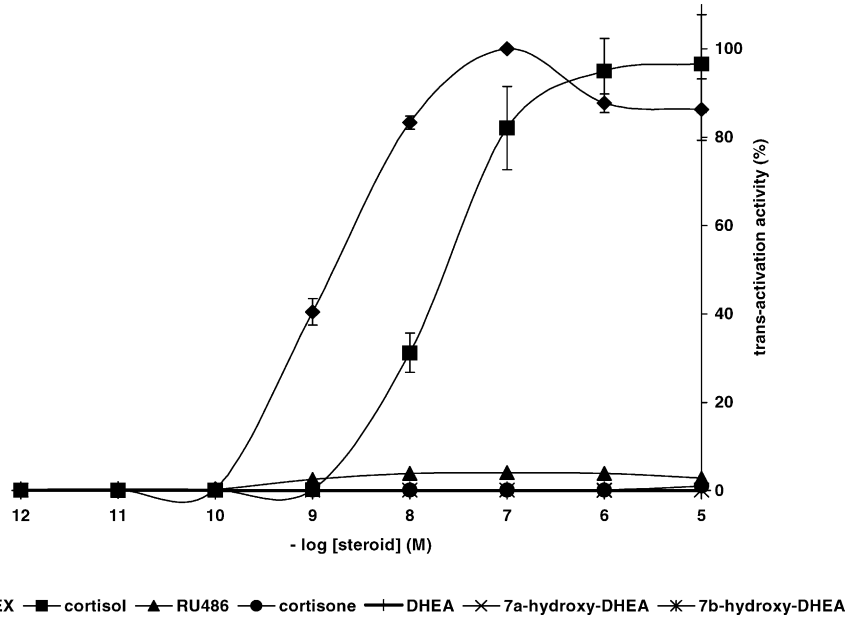

Fig. 1. Investigation of the agonistic activities of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA in transiently transfected COS-7 cells expressing the luciferase-linked hGR. Data are the means ± S.E.M. of triplicate measurements in three independent experiments. The luciferase activity corrected by β-galactosidase control activity is taken as 100% at the maximum of the DEX-induced hGR transactivation activity.

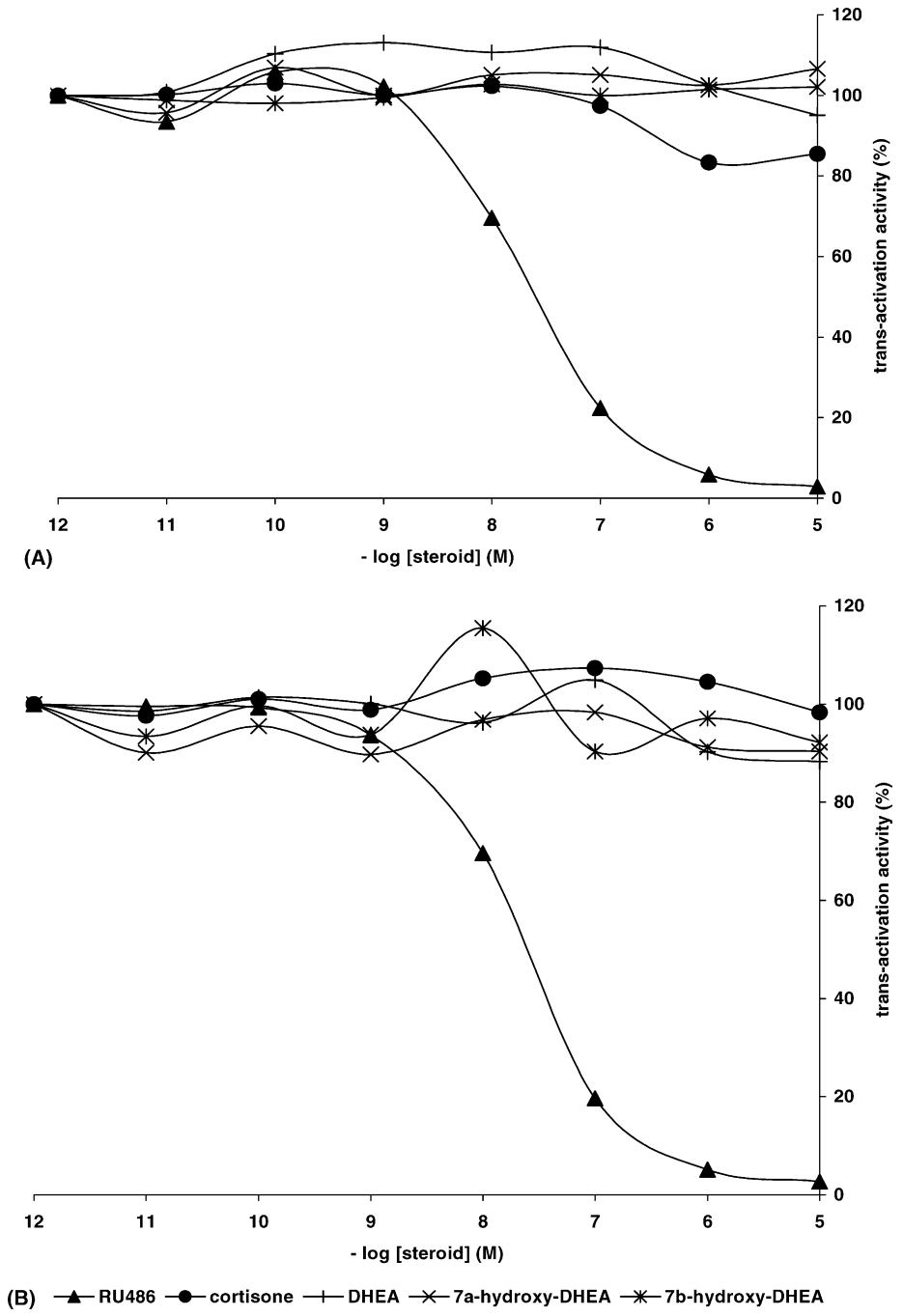

Fig. 2. Investigation of the antagonistic activities of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA in transiently transfected COS-7 cells expressing the luciferase-linked hGR. (A) DEX (10^{-7} M) was the GC used; (B) Cortisol (10^{-6} M) was the GC used. Data are the means of triplicate measurements in two independent experiments. The luciferase activity corrected by β-galactosidase control activity is taken as 100% at the maximum of the GC-induced hGR transactivation activity.

cytoplasm after 60 min (Fig. 4). These patterns were not changed when either 10^{-5} M DHEA, 7α-hydroxy-DHEA, or 7β-hydroxy-DHEA was added to 10^{-8} M DEX, or when 7α-hydroxy-DHEA was added to 10^{-8} M cortisol (Fig. 5). These findings imply that neither DHEA, 7α-hydroxy-DHEA, nor 7β-hydroxy-DHEA interfere with the nuclear-cytoplasmic trafficking of the hGR in the transfected COS-7 cells.

3.3. DHEA, cortisol and 7α-hydroxy-DHEA metabolism in COS-7 cells

As steroid metabolism could be of importance in the COS-7 cells, we investigated the putative transformation of DHEA, cortisol and 7α-hydroxy-DHEA in intact COS-7 cells (10^5 cells per well). Incubations with 10^{-6} M DHEA contained 50,000 dpm of [4-^{14}C]-DHEA; the control incubations had

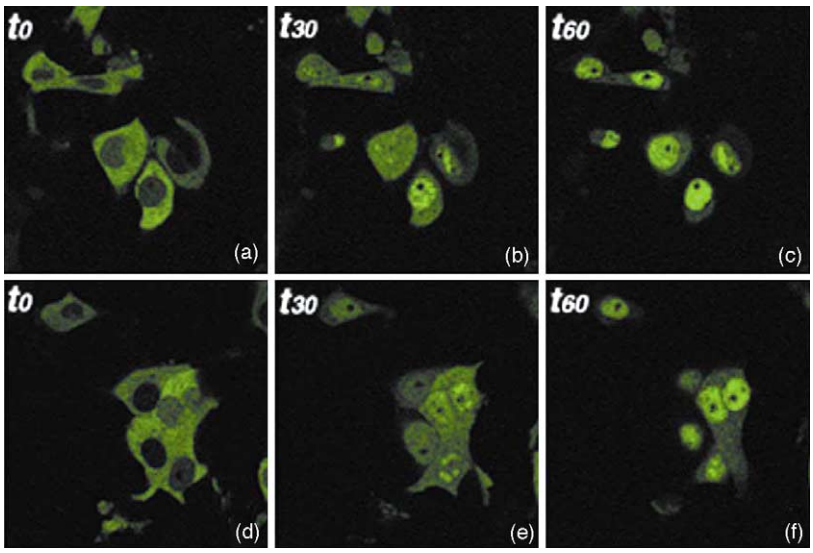

Fig. 3. Investigation of the effect of steroids on the hGR trafficking in transiently transfected COS-7 cells expressing the EGFP-linked hGR. Confocal microscopic examination is reported 0 min (t_0), 30 min (t_{30}) and 60 min (t_{60}) after addition of the steroids. (a–c): addition of 10^{-8} M DEX; (d–f): addition of 10^{-8} M cortisol.

no COS-7 cells in the cultured medium. TLC analysis of ethyl acetate extracts of the media showed that COS-7 cells did not transform DHEA into 7α-hydroxy-DHEA after 24 h. This same experiment was repeated with cortisol and [4-^{14}C]-cortisol; a comparison with control incubations showed no difference, thus COS-7 cells did not oxidize cortisol into cortisone. Another experiment with 7α-hydroxy-DHEA and [4-^{14}C]-7α-hydroxy-DHEA was compared with control in-

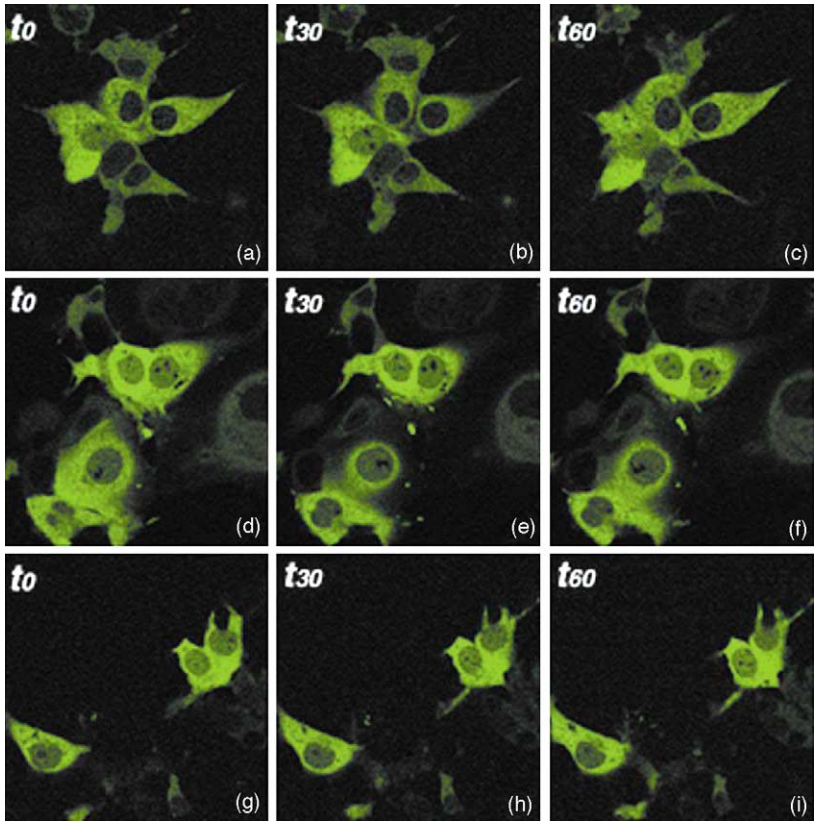

Fig. 4. Investigation of the effect of steroids on the hGR trafficking in transiently transfected COS-7 cells expressing the EGFP-linked hGR. Confocal microscopic examination is reported 0 min (t_0), 30 min (t_{30}) and 60 min (t_{60}) after addition of the steroids. (a–c): addition of 10^{-5} M DHEA; (d–f): addition of 10^{-5} M 7α-hydroxy-DHEA; (g–i): addition of 10^{-5} M 7β-hydroxy-DHEA.

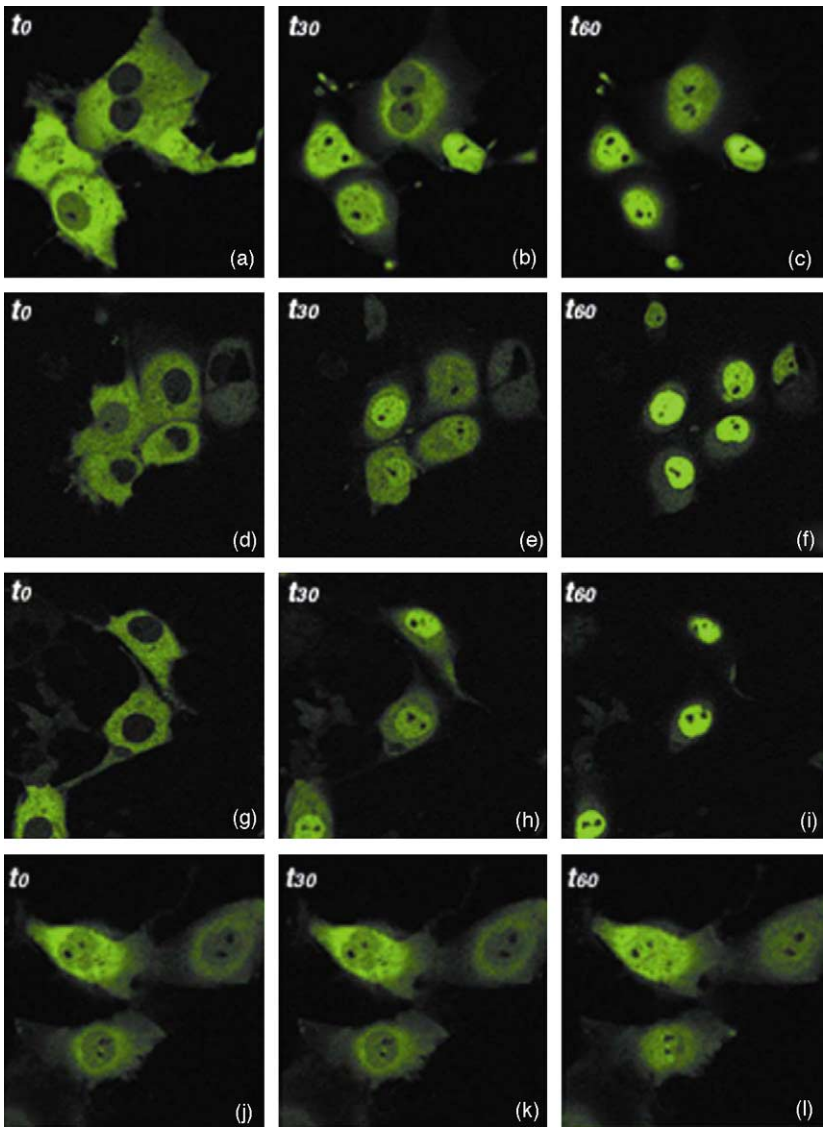

Fig. 5. Investigation of the effect of steroids on the hGR trafficking in transiently transfected COS-7 cells expressing the EGFP-linked hGR. Confocal microscopic examination is reported 0 min (t_0), 30 min (t_{30}) and 60 min (t_{60}) after addition of the steroids. (a, b, c): addition of 10^{-8} M DEX + 10^{-5} M DHEA; (d–f): addition of 10^{-8} M DEX + 10^{-5} M 7α-hydroxy-DHEA; (g–i): addition of 10^{-8} M cortisol + 10^{-5} M 7α-hydroxy-DHEA; (j–l): addition of 10^{-8} M DEX + 10^{-5} M 7β-hydroxy-DHEA.

cubations which showed that after 24 h, less than 2% of the label was found at the level of 7-oxo-DHEA, thus COS-7 cells exert a limited oxidation of 7α-hydroxy-DHEA into 7-oxo-DHEA.

4. Discussion

After the results previously obtained with [^{3}H]-DEX and DHEA derivatives on the GR in several mouse organs [17], our aim was to test whether the hGR transactivation activity and trafficking were modified by DHEA and its 7-hydroxylated derivatives. We selected COS-7 cells for transfection assays because of the hGR cDNA availability and the possibility of transfectable contructs. The decision to use COS-7 cells was influenced by several studies reporting them to be good models for transfections [18,24,25] and not expressing any functional GR [24,26]. Yet other works that had examined the GC metabolism in COS-7 cells yielded contradictory results. One case reported the mock transfected COS-7 cells as neither oxidizing corticosterone into 11-dehydrocorticosterone nor reducing 11-dehydrocorticosterone into corticosterone after 24 h [25]. Another case showed that corticosterone was oxidized into its 11-oxo derivatives after 24 h [27]. Our examination of cortisol metabolism through the use of intact COS-7 cells supported Low's findings [25]. Despite this evidence, cortisol metabolism should not be of importance as our measurements

of the hGR trafficking in all assays were completed after 60 min, and 24 h were required for a significant metabolism of corticosterone [27] or cortisol. Even though insignificant 7α-hydroxylation of DHEA and oxidation of 7α-hydroxy-DHEA was obtained in the COS-7 cells used, the same reasoning applies for the putative metabolite-mediated effects of these steroids. In consideration of the above, the COS-7 cell model was used as a valid tool for our investigations.

Our results obtained from the COS-7 cells transiently transfected with the hGR and luciferase reporter genes showed that neither DHEA nor its 7-hydroxylated metabolites triggered any agonistic or antagonistic effects. We already showed through DEX binding experiments that DHEA, 7α-hydroxy-DHEA, and 7β-hydroxy-DHEA did not compete with DEX binding to the GR in the liver, brain, thymus, and spleen of mice [17], and our present results extend these findings to humans.

Another possible mechanism of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA antiglucocorticoid effects was through interference with the cellular trafficking of the activated hGR. This paradigm was examined previously in cells from mice liver, brain, thymus and spleen cultured in the presence of ^{3}H-DEX and followed by radioactivity measurements in cytoplasmic and nuclear fractions. Indeed, the treatment of cells with 7α-hydroxy-DHEA and 7β-hydroxy-DHEA decreased the nuclear DEX-associated radioactivity levels to larger extents than DHEA treatments [17]. According to the DHEA 7α-hydroxylating capacity in mice cells examined, and because of the absence of affinity of DHEA, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA for the mouse GR, these findings led to the conclusion that the nucleocytoplasmic trafficking of the activated mouse GR was decreased by both 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. Two possible mechanisms could explain these findings: first, one suspects the interference of 7α-hydroxy-DHEA and 7β-hydroxy-DHEA with a protein necessary for the passage of the activated GR through the nuclear membrane; second, the selective interferences of 7α-hydroxy-DHEA and 7β-hydroxy-DHEA with cellular steroid metabolizing enzymes are responsible for the levels of the active GC made available to the cellular GR. Our present findings with COS-7 cells transiently transfected with the hGR-EGFP construct clearly eliminate the first hypothesis since 7α-hydroxy-DHEA and 7β-hydroxy-DHEA treatments did not decrease the hGR trafficking. Since COS-7 cells did not produce any significant amount of cortisone and 7α-hydroxy-DHEA metabolites after 24 h, these cells were fully adequate for the test of that first hypothesis. In contrast, our COS-7 model was not adequate for the testing of the second hypothesis. One report brings support to it: when showing in hepatocytes that 7α-hydroxy-DHEA was a substrate for the 11β-HSD1 responsible for cortisone or 11-dehydrocorticosterone activation into cortisol and corticosterone [15].

It is then possible that the antiglucocorticoid effects observed with 7α-hydroxy-DHEA in vivo and in cells that are targets for GC (such as hepatocytes, neurons and lymphocytes [17,28]), result from its interferences [15] with the 11β-HSD1-mediated activation of cortisone or 11-dehydrocorticosterone into active GC. Investigation of this prospect needs other experimental approaches, including a thorough study of the 11β-HSD1 cross-inhibitions by GC and 7α-hydroxy-DHEA.

Acknowledgements

This work is a part of the thesis of Caroline Muller, and was supported by a grant from Hunter–Fleming Inc. (UK). The authors wish to thank Patrice Rool (Roowin SA, Paris, France) for the gift of the 7-hydroxylated DHEA derivatives.

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