STUDIES ON THE MECHANISM OF ANTIGLUCOCORTICOID ACTION OF 7α-HYDROXYDEHYDROEPIANDROSTERONE

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Dedicated to Dr Jan Fajkos on the occasion of his 75th birthday.

The literature reports that 7α-hydroxydehydroepiandrosterone (7α-OH-DHEA), a metabolite of dehydroepiandrosterone (DHEA), displays numerous anti-aging properties (such as immunostimulation and anti-apoptotic effects) which may result from an apparent antiglucocorticoid activity. However, the molecular mechanism(s) of this effect remain to be elucidated. In the present work, we attempted to unravel some aspects of this mechanism *in vitro*, in adrenalectomized rats. No specific binding of [^{3}H]-7α-OH-DHEA occurred with the hepatic cytosolic fraction, and the binding of [^{3}H]-dexamethasone ([^{3}H]-DEX) to the cytosolic glucocorticoid receptor complex (GCRC) was unaffected by increasing concentrations of either DHEA or 7α-OH-DHEA. In marked contrast, in isolated hepatic nuclei, the retention of partially purified [^{3}H]-DEX-labelled cytosolic GCRC was significantly decreased after nuclei preincubation with 7α-OH-DHEA, DHEA or 7α-hydroxypregnenolone. However, further experiments using isolated cytosolic fraction preactivated with [^{3}H]-DEX and then filtered on DNA-cellulose columns in the presence or in the absence of 7α-hydroxy steroids unequivocally demonstrated that 7α-OH-DHEA neither competed with the activation of the GCRC, nor inhibited the binding of this complex to DNA-cellulose in the cell-free system. The effect of 7α-OH-DHEA on membrane fluidity of brain cell membranes was observed only at concentrations higher that that of the parent substance DHEA. Thus, the effect of 7α-OH-DHEA does not seem to be mediated by the influence of the accessibility of the hormone to intracellular receptors. While the GCRC binding to DNA is apparently unaffected by 7α-OH-DHEA, and cannot therefore explain the lesser retention of DEX-activated GCRC in isolated nuclei, other mechanisms, possibly extranuclear, such as modification of the conformation of GCRC may be involved. The GCRC in the presence of 7α-OH-DHEA, may account for the antiglucocorticoid properties of this steroid which are currently under investigation.

Key words: 7α-Hydroxydehydroepiandrosterone; Dehydroepiandrosterone; Glucocorticoid receptor; Membrane fluidity; Antiglucocorticoid effect; Steroids.

Though dehydroepiandrosterone sulfate, 17-oxoandrost-5-en-3β-yl sulfate (DHEAS), is the most abundant steroid in human blood, there is still little information on its true biological activity, despite many studies demonstrating association of DHEAS levels with various physiological and pathological situations. The revival interest for DHEAS and free dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one (DHEA), by various investigators claiming many beneficial metabolic effects of DHEA and suggesting that this steroid might be a "fountain of youth" was recently reviewed[1,2]. Among its many biological effects, DHEA displays some activities which can be defined as opposing the effects of glucocorticoids[3].

Using animal models as well as immunocompetent cells, experimental evidence has been accumulated that DHEA counteracted the immunosuppressive effects of glucocorticoids. In brief, DHEA changed the patterns of glucocorticoid induced interleukin production, counteracted glucocorticoid-induced suppression of both T- and B-lymphocyte proliferation and of antibody production, inhibited thymic involution, acted against development of hypertension in rats and impaired differentiation of fibroblasts into adipocytes[3]. DHEA also blocked glucocorticoid-induced activation and gene expression of several key enzymes of carbohydrate and amino acid metabolism, *e.g.* glucose-6-phosphate dehydrogenase, tyrosine aminotransferase or ornithine decarboxylase[3].

The fact that relatively large doses of DHEA are required to demonstrate its immunoprotective effects[4–6] rises a question whether not just DHEA itself, but some of its metabolites are responsible for these actions[5,6]. Recent reports indicate that 7-hydroxylated derivatives of DHEA may be such candidates. The hypothesis that 7-hydroxylated metabolites of DHEA act as potent endocrine regulators of the immune response has been tested first by Padgett and Loria[5] who compared various DHEA metabolites as to their ability to confer resistance to bacterial or viral infection, and by Morfin and Courchay[6] who showed that production of anti-lysozyme immunoglobulins in mice was triggered by much lower doses of 7α-hydroxy-DHEA; 3β,7α-dihydroxyandrost-5-en-17-one (7α-OH-DHEA), than were the necessary doses of DHEA.

Human PBMCs and murine T cells express glucocorticoid receptors. The deleterious effect of glucocorticoids on these cells is well documented and shows that these hormones are involved in the process of immune cell death through apoptosis. Indeed, when murine thymic cells are cultivated in the presence of both dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione (DEX), and 7α-OH-DHEA, glucocorticoid-induced apoptosis was decreased to a larger extent than with DHEA (ref.[7]). Recently, Loria[8] pointed to different effects of DHEA derivatives, androst-5-ene-3β,17β-diol and androst-5-ene-3β,7β,17β-triol, on the regulation of host immune response after infection and stress-induced immunosuppression. Only the latter steroid, a native 7β-hydroxylated metabolite of DHEA was able to potentiate markedly the cellular response by increasing lymphocyte activation and counteracting the immu-

nosuppressive activity of corticosterone on lymphocyte proliferation and cytokine production.

The mechanism of antiglucocorticoid action of 7α-OH-DHEA is yet unknown. In the target cell, the glucocorticoid (GC) binds to its receptor (GR) forming a cytoplasmic complex (GRGC). GRGC is then translocated into the nucleus[9,10]. Both in cell-free systems and in intact cells, this translocation requires a process termed "activation" whereby the GRGC acquires ability to bind to DNA (refs[9,10]). In an elegant study addressing the mechanism of GRGC activation in rat thymic cells[11] it was found that activation is associated with a significant decrease in the apparent molecular weight of the GRGC. This is usually explained by the dissociation of heat shock proteins from the GRGC. Activated GRGC (100 kDa) could be separated from its non-activated form (330 kDa) due to an increased affinity of the activated complex for DNA-cellulose. After passage through a DNA-cellulose column, the non-activated GRGC can be retained on another column packed with DEAE-cellulose, and the remaining fragments, displaying affinity neither for DNA-cellulose nor for DEAE-cellulose, designated usually as "mero-receptors" (27 kDa) can be finally retained on a hydroxyapatite column. The activated GRGC forms a homodimer with "zinc fingers" which binds to palindromic DNA sequences in the promoter region of cognate gene(s) known as glucocorticoid responsive elements. This binding triggers then a sequence of events starting by interaction(s) of the DNA-bound GRGC with other nuclear transcription factors which, in turn, result in initiation or suppression of transcription of the regulated gene[12]. All of these events involved in the processes of transcription and translation may be modulated by various agents, including steroids.

In the present study, we investigated the effect of 7α-OH-DHEA on the first steps of the outlined mechanism, namely whether 7α-OH-DHEA and related steroids might influence the events following the binding of GC to GR (*e.g.*, either activation of the GRGC or binding of the activated GRGC to DNA), using the *in vitro* technique described above. Besides, it was shown in murine liver cells that 7α-OH-DHEA decreased the retention of [^{3}H]-DEX in the nuclear cell fraction[13]. To ascertain this effect herein, experiments were designed for producing [^{3}H]-DEX-activated cytosolic GRGC and for assay of its retention in isolated hepatic nuclei from adrenalectomized rats in the presence or absence of 7α-hydroxylated steroids.

Finally, one of the early stages of the immune response involves a reorganization of cell membrane constituents resulting in changes of influx and release of various compounds (*e.g.*, of ions, substrates, hormones, mediators). These changes are reflected by an altered membrane fluidity, which can be measured by various physicochemical methods[14]. Therefore, the effect of 7α-OH-DHEA and of other steroids of the 3β-hydroxy-5-ene series on the membrane fluidity of the rat brain cell membrane has been measured by the fluorescence polarization technique.

EXPERIMENTAL

Steroids and Reagents

All reagents were from Sigma–Aldrich (St. Louis, MO, U.S.A), except for 11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(1-propenyl)estra-4,9-dien-3-one (RU486) which was a gift from Roussel–Uclaf (Romainville, France). 7α-OH-DHEA was prepared as previously described[15] and other 7α-hydroxylated steroids were from the collection of one of us (R. M.). [1,2-^{3}H]-DHEA (54.9 Ci/mmol) and [6,7-^{3}H]-DEX (43.9 Ci/mmol) were NEN products purchased from Dupont de Nemours S.A. (France). [1,2-^{3}H]-7α-OH-DHEA (54.9 Ci/mmol) was prepared as described[16] using mouse brain microsomes with [1,2-^{3}H]-DHEA. The ^{3}H-labelled 7α-OH-DHEA produced was isolated by thin layer chromatography and further purified by high performance liquid chromatography on a 25 cm Chromasil C_{18} reverse phase column eluted with methanol–water (7 : 3, v/v). Radiochemical purity of the [1,2-^{3}H]-7α-OH-DHEA was measured at 99.1% by crystallization to constant specific activity of a carrier-diluted portion.

Preparations of Cytosol and Nuclei

The method for preparation of cytosol from rat tissues[17] was used with slight modifications. Briefly, adrenalectomized male Wistar rats were anesthetized with diethyl ether and exsanguinated through the abdominal aorta. Livers were quickly perfused with ice-cold 0.9% NaCl through the portal vein. Organs (liver and brain) were collected and rinsed in ice-cold Tris-glycerol (TG) buffer, pH 7.4 (10 mM Tris-HCl, 10% (v/v) glycerol, 1.5 mM EDTA, 2 mM DTT, 20 mM sodium molybdate, 1 mM PMSF, aprotinin 1 μg/ml, leupeptin 1 μg/ml). Organs were then weighted and homogenized at 4 °C in 3 volumes of TG buffer in a Potter–Elvehjem homogenizer. Subsequently, homogenates were centrifuged (12 000 *g* for 20 min at 4 °C) and supernatants were further centrifuged (105 000 *g* for 60 min at 4 °C). The last supernatant (cytosol fraction) was immediately stored at –70 °C.

Cytosol from human PBMCs was prepared as follows: 100 ml of blood was applied on a Ficoll-verografin layer and centrifuged in a density gradient (1 000 *g* for 10 min at 4 °C). The thin intermediate layer of PBMCs was carefully collected, resuspended in ice-cold PBS and centrifuged again (1 000 *g* for 10 min at 4 °C). The pellet was resuspended in PBS (1 : 1, v/v) and rapidly frozen at –70 °C for storage. Cytosols obtained from these cell preparations were thawed once at 2 °C, homogenized in 10 volumes of TG buffer and centrifuged as above. The cytosols of PBMCs were used immediately after preparation.

Rat liver nuclei were isolated according to the method of Leake and Habib[18]. Briefly, livers isolated and rinsed as above were homogenized in STKM buffer, pH 7.4 (50 mM Tris-HCl, 0.25 M saccharose, 25 mM KCl, 5 mM $MgCl_2$, extemporaneously added with 4 mM PMSF and leupeptin 10 μg/ml). Following a first centrifugation (5 000 *g* for 5 min at 4 °C), the resulting pellet was resuspended in STKM buffer added with 0.1% triton X100, rehomogenized and centrifuged again (800 *g* for 10 min at 4 °C). The nuclear pellet was washed twice in STKM buffer (800 *g* for 10 min at 4 °C) and stored at –70 °C until further use[19]. The method of Burton[20] was used for nuclear DNA measurements.

Cytosolic Binding Assays

For investigations of a putative cytosolic binding of 7α-OH-DHEA, [1,2-^{3}H]-7α-OH-DHEA ($1 . 10^{-9}$, $5 . 10^{-9}$ and $1 . 10^{-8}$ M) was incubated with 0.2 ml of rat liver cytosol for 24 h at 4 °C. In order to determine non-specific binding, each incubation was repeated with a 500-fold excess of non-radiolabelled 7α-OH-DHEA. All incubations were carried out in triplicate. After the incubation, free and bound fractions were separated by using the dextran–charcoal technique[18]. Radioactivity of the bound

fraction was measured by counting of supernatants, and specific binding was determined by substracting non-specific binding from the total one. Investigations for 7α-OH-DHEA putative interference with cytosolic binding of glucocorticoids, [6,7-^{3}H]-DEX ($1 . 10^{-8}$ M) was incubated with 0.2 ml of rat liver cytosol for 24 h at 4 °C. Increasing concentrations of either DEX or corticosterone or DHEA or RU486 or 7α-OH-DHEA (0, $1 . 10^{-8}$, $1 . 10^{-7}$, $1 . 10^{-6}$, $1 . 10^{-5}$ M) were added. All incubations were carried out in triplicate. Free and bound fractions were separated and counted as described above.

Activation of the Cytosolic Glucocorticoid Receptor

In a typical experiment, cytosol was mixed with an equal volume of TAPS buffer, pH 8.8 (25 mM TAPS-sodium salt, 1 mM EDTA, glycerol 10%, v/v) and [^{3}H]-DEX (16 pmol/ml cytosol) was added, with or without the non-radioactive steroids tested. The mixture was incubated in an ice bath for 150 min followed by heat activation for 30 min at 25 °C. The heat-activated [^{3}H]-DEX-labelled GRGC was used for further experiments. In other experiments, addition of the non-radioactive steroids followed the heat-activation step. In some instances, the heat-activated [^{3}H]-DEX-labelled GRGC was further separated on a refrigerated Sephadex G25 microcolumn (bed height 2 cm) eluting with TAPS buffer. The GRGC-containing protein fraction was used immediately in experiments described below. Non-specific binding of [^{3}H]-DEX was determined from identical incubations containing 100-fold molar excess of DEX.

Measurement of Activated Cytosolic Glucocorticoid Receptor Species

For separation of the activated GR from unbound [^{3}H]-DEX and non-activated GR, [^{3}H]-DEX-labelled heat-activated cytosol, either with or without further processing on Sephadex G25, was applied on to a DNA-cellulose column (200 mg of dry DNA-cellulose per column) connected with a second column filled with DEAE-cellulose (bed height 10 mm) and eluted with 8 ml TAPS buffer. For column use, pipette plastic tips (5 ml, Fintip 62 from Labsystems, France) were used with glass wool at the bottom and silicon tubing for flow control. All column packings were suspended in the TAPS buffer before loading. Twelve columns were mounted on a solid-phase extraction manifold (Supelco, Bellefonte, PA, U.S.A.). Under gentle vacuum, elution time ranged from 6 to 10 min. The eluate, containing mero-receptors and unbound DEX, was applied onto a third column filled with hydroxyapatite (bed height 6 mm) and mounted on the manifold apparatus.

Incubation of Isolated Nuclei with the Activated GRGC

[^{3}H]-DEX-Labelled activated GRGC was obtained as described above. In plastic Eppendorf tubes, thawed and gently suspended nuclei (6–10 μg of nuclear DNA) were preincubated with or without unlabelled steroids in HEPES/EDTA buffer (pH 7.6) for 20 min at 37 °C. The GRGC suspension (40 000 dpm of [^{3}H]-DEX) was added, the volume completed to 0.2 ml with buffer, and incubation was continued for additional 20 min at 37 °C. Incubations were stopped by immersing the tubes into an ice bath followed by the immediate centrifugation at 1 000 *g* for 5 min at 4 °C. The resulting nuclear pellets were then washed twice with HEPES/EDTA buffer and digested in 0.2 ml of 1 M sodium acetate under intense shaking for 60 min at room temperature[21]. Nuclear GRGC-associated [^{3}H]-DEX of the digests was counted by liquid scintillation spectrometry (Intertechnique SL-4000, Kontron, Switzerland).

Studies of the Effect of Steroids on Membrane Fluidity

Crude membrane preparation: Male Wistar rats aged 55–65 days, were killed by decapitation. Homogenates of the brain tissue in ice-cold 50 mM Tris-HCl (pH 7.4) were filtered through six layers of surgical gauze, centrifuged at 1 000 *g* for 15 min and the supernatants were further centrifuged[22] at 20 000 *g* for 30 min. The final pellets were resuspended in the same buffer (200 mg tissue per ml).

Incubation of tissues with steroids: 3 mg of each steroid was dissolved in DMSO (0.2 ml). On stirring the solution, the volume was adjusted with Tris-HCl (pH 7.4) + 3.5% PVP to 10 ml. The dispersion was briefly sonicated (15 s, 30 W). The crude brain membrane fraction in Tris-HCl buffer (1.5 ml) was incubated under stirring for 90 min with various concentrations of steroid suspensions at 25 °C. Controls were incubated with the same concentration of PVP and DMSO in Tris buffer[23,24]. The brain membranes were then centrifuged at 20 000 *g* for 30 min, and the pellets were suspended in Tris-HCl buffer (200 mg/ml).

Fluorescence polarization measurements: Fluorescence polarization was measured with a Perkin–Elmer LS-5 luminescence spectrometer, equipped with circulation bath to maintain the sample temperature[25] at 25 °C. A solution of 2 mmol DPH in tetrahydrofuran was dispersed by 1 000-fold agitative dilution in 50 mmol/l Tris-HCl buffer. Crude brain membranes (100 μg protein) were incubated at 25 °C for 1 h with 2 ml DPH in Tris-buffer saline. The fluorescence polarization was computed by the equation

$$P = [I_{vv} - I_{vh}(I_{hv}/I_{hh})]/[I_{vv} + I_{vh}(I_{hv}/I_{hh})],$$

where I_{vv} and I_{hh} are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular to the direction of vertically polarized light. I_{hv}/I_{hh} is the ratio when the excitation is polarized horizontally and the emission observed through the analyzer oriented perpendicularly and in parallel, respectively. Lipid microviscosity was estimated by the empirical relation[23], $2P/(0.46 - P)$.

Statistical Analysis

The best estimation of the mean values, variances and between group comparisons were performed by the robust Horn pivot method[26]. The statistical significance of Pearson's correlation coefficients was evaluated by the F-test. The robust tests used for determination of significance are also described in Tables IV and V.

RESULTS

Cytosolic Binding Studies

Cytosolic preparations from liver of adrenalectomized rats were incubated in triplicate with [^{3}H]-7α-OH-DHEA ($1 . 10^{-9}$–$1 . 10^{-8}$ M), and without a 200-fold molar excess of unlabelled 7α-OH-DHEA. Throughout 3 experiments, no specific binding was obtained (data not shown).

Binding of [^{3}H]-DEX ($1 . 10^{-8}$ M = 10 Kd) to rat liver cytosol was assayed in the presence of each of three known competitors for this binding, DEX, RU486 and corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione), or in the presence of DHEA or 7α-OH-DHEA. Each of these steroids was tested at the following concentrations: $1 . 10^{-8}$, $1 . 10^{-7}$, $1 . 10^{-6}$ and $1 . 10^{-5}$ M. Results are reported in Table I. For each steroid, effects

of the concentrations tested were calculated together for a general ANOVA comparison using Statgraph® and were expressed as global means ± s.e.m. Unlabelled DEX, RU486 and corticosterone significantly competed with [^{3}H]-DEX binding. However, neither DHEA nor 7α-OH-DHEA displaced significantly the binding of [^{3}H]-DEX to its receptor. These data clearly document absence of interaction of DHEA and 7α-OH-DHEA with the binding of glucocorticoids to their cytosolic receptor.

Nuclear Retention of Activated GRGC

Results of the retention experiments are shown in Table II. Retention of [^{3}H]-DEX-activated GRGC by isolated rat liver nuclei was significantly decreased after preincubation of the nuclei with 7α-OH-DHEA or with 7α-OH-pregnenolone (7α-OH-PREG, 3β,7α-dihydroxypregn-5-en-20-one), and was decreased to a lesser extent after preincubation with DHEA or with pregnenolone (PREG, 3β-hydroxypregn-5-en-20-one). No such effect was obtained upon simultaneous incubation with both [^{3}H]-DEX-activated GRGC and the tested unlabelled steroids (data not shown). Significant differences ($p < 0.01$) were found between the effects of 7α-OH-DHEA and those of 7α-OH-pregnenolone, as well as between DEX and DHEA on one hand and DEX and pregnenolone on the other hand. The effects of DHEA *versus* 7α-OH-DHEA and those of pregnenolone *versus* 7α-OH-pregnenolone were not significantly different.

TABLE I
Binding of [^{3}H]-DEX to rat liver cytosol in the absence or in presence of unlabelled steroids[a]

Conditions	dpm/100 µl supernatant (mean ± s.e.m.)	*n*	Statistical differences *versus* control
Control [^{3}H]-DEX 5 . 10^{-8} M	1 368 ± 81	17	–
Dexamethasone	400 ± 74	31	$p < 0.001$
Corticosterone	425 ± 93	12	$p < 0.001$
RU 486	341 ± 119	12	$p < 0.001$
DHEA	1 211 ± 92	29	[b]
7α-OH-DHEA	1 425 ± 81	30	[b]

[a] Unlabelled steroids were incubated overnight with cytosol samples activated with [^{3}H]-DEX (1 . 10^{-8} M) from livers of adrenalectomized rats as described in Experimental, the unbound steroids were then removed upon treatment with the activated dextran–charcoal. For each steroid, the data herein are means of the effects of the 4 concentrations tested (1 . 10^{-8}, 1 . 10^{-7}, 1 . 10^{-6} and 1 . 10^{-5} M). Each experiment was repeated 3 times and was performed in triplicate or in quadruplicate. [b] Non significant.

Retention of GRGC on DNA-Cellulose after Preincubation with 7α-Hydroxy Steroids

In the first series of experiments, the effects of preincubation with 7α-OH-DHEA was tested on the retention of heat-activated [^{3}H]-DEX-labelled cytosol (without Sephadex G25 purification) on each of the three columns connected in a series and filled with DNA-cellulose, DEAE-cellulose and hydroxyapatite, respectively. Cytosols from rat liver, rat brain and human PBMCs were used. Each experiment was repeated six times. The results are summarized in Table III. No significant difference in retention on any column was found when cytosol was incubated with or without a 100-fold excess of 7α-OH-DHEA. Measurement of non-specific binding of [^{3}H]-DEX resulted from incubations in the presence of a 100-fold excess DEX. For rat liver cytosol, non-specific binding on DNA-cellulose, DEAE-cellulose and hydroxyapatite were 2.94 ± 1.43, 19.1 ± 5.85 and 96.47 ± 12.10 per cent of [^{3}H]-DEX binding measurements, respectively.

TABLE II
Effects of dehydroepiandrosterone (DHEA), pregnenolone (PREG), 7α-OH-DHEA and 7α-OH-PREG on the retention of [^{3}H]-DEX-preactivated glucocorticoid receptor (GRGC) in isolated nuclei from rate liver[a]

Preincubation conditions	dpm ± s.e.m.	*n*	Statistical differences *versus*	
			control	other compound
Control [^{3}H]-DEX ($5 . 10^{-8}$ M)	1 043 ± 58	16		
		$500 . 10^{-8}$ M steroids added		
DEX	736 ± 35	8	$p < 0.001$	
DHEA	560 ± 51	9	$p < 0.001$	DEX, $p < 0.001$; 7α-OH-DHEA, NS
7α-OH-DHEA	469 ± 15	4	$p < 0.001$	7α-OH-PREG, $p < 0.01$
PREG	725 ± 37	4	$p < 0.001$	DHEA, $p < 0.01$; 7α-OH-PREG, NS
7α-OH-PREG	601 ± 79	4	$p < 0.001$	
		$1\,000 . 10^{-8}$ M steroids added		
DEX	535 ± 82	9	$p < 0.001$	
DHEA	326 ± 62	9	$p < 0.001$	DEX, $p < 0.001$; 7α-OH-DHEA, NS
7α-OH-DHEA	457 ± 58	12	$p < 0.001$	7α-OH-PREG, $p < 0.01$
PREG	714 ± 29	10	$p < 0.001$	DHEA, $p < 0.01$; 7α-OH-PREG, NS
7α-OH-PREG	635 ± 46	9	$p < 0.001$	

[a] Isolated hepatic nuclei from adrenalectomized rats were first incubated with the unlabelled steroids and then further incubated with the [^{3}H]-DEX-preactivated GRGC as detailed in the Experimental. Each result is the mean of 3 different experiments made in triplicate or in quadruplicate. Statistical differences were assessed by ANOVA (NS, not significant).

In the second series of experiments, the effect of increasing doses of 7α-OH-DHEA (from 10- to 500-fold excess relative to the [^{3}H]-DEX concentration) was tested on the retention of [^{3}H]-DEX-labelled heat-activated GRGC from rat liver cytosol. The same experimental design as in the first experiment was used with the exception of passage through hydroxyapatite. As shown in Table IV, no significant correlation was found between the retention of [^{3}H]-DEX labelled activated GRGC on either DNA-cellulose or DEAE-cellulose, and 7α-OH-DHEA doses.

The third series of experiments differed from the previous ones in that the Sephadex G25 purification of the activated GRGC was included prior to application on the columns retaining individual GRGC fractions. Also, 7α-OH-DHEA was added only after heat-activation. Increasing amounts of 7α-OH-DHEA (30- to 300-fold molar excess relative to [^{3}H]-DEX concentration) were tested. As demonstrated in Table V, no signi-

TABLE III

The effect of 7α-OH-DHEA on the binding of dexamethasone-labelled cytosols from two rat and one human tissue to the matrixes retaining various forms of glucocorticoid–glucocorticoid receptor complex (GRGC)[a]

Cytosol and	DNA-cellulose	DEAE-cellulose	Hydroxyapatite
		Rat livers	
[^{3}H]-DEX	64.9 ± 2.3	58.6 ± 27.5	31.2 ± 3.5
[^{3}H]-DEX + 7α-OH-DHEA	64.5 ± 3.3	55.6 ± 13.2	29.7 ± 3.4
[^{3}H]-DEX + 100-fold excess of DEX	1.91 ± 0.93	11.2 ± 3.43	30.1 ± 4.1
		Rat brain	
[^{3}H]-DEX	10.2 ± 3.5	25.5 ± 8.8	48.5 ± 5.8
[^{3}H]-DEX + 7α-OH-DHEA	9.3 ± 2.8	35.5 ± 20.6	58.0 ± 17.8
[^{3}H]-DEX + 100-fold excess of DEX	1.93 ± 0.55	6.02 ± 2.71	51.6 ± 7.6
		Human PBMC	
[^{3}H]-DEX	4.79 ± 1.70	25.5 ± 8.8	48.5 ± 5.8
[^{3}H]-DEX + 7α-OH-DHEA	4.81 ± 0.15	35.5 ± 20.6	58.0 ± 17.8
[^{3}H]-DEX + 100-fold excess of DEX	2.18 ± 0.23	[b]	[b]

[a] The cytosolic fractions from homogenates of rat livers, rat brains and human PBMC were pre-incubated with [^{3}H]-DEX in the presence or absence of 100-fold molar excess of 7α-OH-DHEA, heat-activated and applied successively on the columns filled with DNA-cellulose retaining activated GRGC, DEAE-cellulose retaining non-activated GRGC, and hydroxyapatite retaining meroreceptors. Non-specific binding was determined by measuring retention of [^{3}H]-DEX in the presence of non-radioactive DEX in a 100-fold molar excess. The values represent amounts (in fmol) of retained [^{3}H]-DEX, means from six parallel determinations ±95% confidence interval. [b] Not determined.

ficant correlation was found between 7α-OH-DHEA concentrations used and GRGC binding in any of the columns.

In the last set of experiments, the effects of several 3β-hydroxy-5-ene C_{19} and C_{21} steroids on the binding of [^{3}H]-DEX-labelled GRGC onto the columns were tested as above. Steroids were used in a 250-fold molar excess of the [^{3}H]-DEX concentration. Because slightly different quantities of the [^{3}H]-DEX-labelled GRGC were used for each experiment, and in order to make valid comparisons, all column retention data were related to the average retention of [^{3}H]-DEX-labelled GRGC in the absence of the steroids tested. As shown in Table VI, none of the steroid tested affected column retention of [^{3}H]-DEX. In addition, if the mean values of retention on each of the three columns summed (Table VI), 100% was not accurately obtained. This was due to the Horn method used for estimation of the mean value and of the variance. Thus, the mean values obtained differed from the arithmetic mean, especially in experiments with a low number of replicates (n = 4).

TABLE IV
Dependence of GRGC binding to DNA- and DEAE-cellulose on the dose of 7α-OH-DHEA[a]

Test	Retention on DNA-cellulose	Retention on DEAE-cellulose
	Residual analysis	
Cook–Weisberg test of heteroskedasticity of residua	residua are homoskedastic (p < 0.466)	residua are homoskedastic (p < 0.685)
Jarque–Berra test of normality of residual distribution	residua have the Gaussian distribution (p < 0.480)	residua have the Gaussian distribution (p < 0.501)
Wald test of autocorrelation of residua	residua are not autocorrelated (p < 0.079)	residua are not autocorrelated (p < 0.933)
	Results (parameters of the regression ± its 95% confidence interval)	
Intercept	196 ± 3.38 (p < 0.001)	23.7 ± 4.84 (p < 0.001)
Slope	–0.000677 ± 0.0148 (p < 0.927)	–0.00114 ± 0.021 (p < 0.941)
Correlation coefficient	0.0194	0.000677
Fisher–Snedecor F-test	correlation is not significant (p < 0.927)	correlation is not significant (p < 0.914)

[a] Rat liver cytosol was pre-incubated with [^{3}H]-DEX alone or with increasing concentrations of 7α-OH-DHEA (10-, 50-, 100- and 500-fold molar excess). After heat activation, it was applied successively on the columns filled with DNA- and DEAE-cellulose, respectivelly, and the retention of [^{3}H]-DEX was measured. For each concentration of 7α-OH-DHEA five parallel determinations were carried out. The binding as measured by [^{3}H]-DEX retention to the appropriate matrix was correlated with the concentration of 7α-OH-DHEA by using robust statistical tests.

Membrane Fluidity

As shown in Table VII, the microviscosity of rat-brain cell membranes was increased by cholesterol hemisuccinate only in a dose-dependent manner, while DHEA acetate, pregnenolone and its acetate, used at higher concentrations, caused a decrease in membrane fluidity. The 7α-OH-DHEA-triggered decrease of brain cell membrane fluidity was smaller than that induced by the parent non-hydroxylated steroid.

TABLE V
Dependence of the binding of partially purified GRGC to the matrixes retaining various forms of GRGC on the dose of 7α-OH-DHEA[a]

Test	Retention on DNA cellulose	Retention on DEAE cellulose	Retention on hydroxyapatite
	Residual analysis		
Cook–Weisberg test of heteroskedasticity of residua	residua are homoskedastic ($p < 0.194$)	residua are homoskedastic ($p < 0.660$)	residua are homoskedastic ($p < 0.474$)
Jarque–Berra test of normality of residual distribution	residua have the Gaussian distribution ($p < 0.528$)	residua have the Gaussian distribution ($p < 0.736$)	residua have the Gaussian distribution ($p < 0.383$)
Wald test of autocorrelation of residua	residua are not autocorrelated ($p < 0.560$)	residua are not autocorrelated ($p < 0.200$)	residua are not autocorrelated ($p < 0.688$)
	Results (parameters of the regression ± its 95% confidence interval)		
Intercept	103 ± 4.58 ($p < 0.001$)	22.4 ± 2.98 ($p < 0.001$)	9.44 ± 1.17 ($p < 0.001$)
Slope	–0.0084 ± 0.0299 ($p < 0.534$)	–0.00511 ± 0.0195 ($p < 0.562$)	–0.00337 ± 0.00762 ($p < 0.337$)
Correlation coefficient	0.224	0.209	0.339
Fisher–Snedecor F-test	correlation is not significant ($p < 0.534$)	correlation is not significant ($p < 0.562$)	correlation is not significant ($p < 0.337$)

[a] Rat liver cytosol was pre-incubated with [^{3}H]-DEX, heat-activated, then increasing amounts of 7α-OH-DHEA (30-, 60-, 150- and 300-fold molar excess related to DEX) were added and the mixture was purified on a Sephadex G-25 column. The protein fraction was then applied on the system of columns retaining various forms of GRGC as described in Table I. For each concentration of 7α-OH-DHEA two parallel determinations were performed. The GRGC binding to each matrix as measured by retention of [^{3}H]-DEX was correlated with the 7α-OH-DHEA dose by using robust statistical tests.

TABLE VI

The effect of five 3β-hydroxy-5-ene C_{19}- and C_{21}-steroids on the specific binding of dexamethasone-labelled rat liver cytosol to the matrixes retaining various forms of GRGC[a]

Cytosol and	DNA-cellulose	DEAE-cellulose	Hydroxyapaptite
[^{3}H]-DEX	84.0 ± 1.85	15.3 ± 3.25	3.66 ± 0.090
[^{3}H]-DEX + pregnenolone	84.0 ± 4.44	14.7 ± 2.37	4.12 ± 1.10
[^{3}H]-DEX + DHEA	80.7 ± 4.07	15.6 ± 3.47	3.52 ± 0.433
[^{3}H]-DEX + 7α-OH-DHEA	82.4 ± 7.60	15.4 ± 2.65	4.58 ± 1.94
[^{3}H]-DEX + 7α-OH-pregnenolone	80.5 ± 7.23	15.1 ± 4.75	3.68 ± 1.16
[^{3}H]-DEX + 7α-OH-DHEA-3β-palmitate	84.6 ± 8.64	16.3 ± 7.87	3.60 ± 0.955

[a] Rat liver cytosol was pre-incubated with [^{3}H]-DEX, heat activated and mixed with the steroid tested in 250-fold molar excess (related to DEX concentration) and then purified on the SEPHADEX G-25 column. The protein fraction was then applied on the system of columns retaining various forms of GRGC as described in Tables I and III and retention of [^{3}H]-DEX was measured. Values are expressed as per cents of [^{3}H]-DEX retained in the absence of competitors, the means ± their 95% confidence intervals from four parallel measurement are given.

TABLE VII

Changes in the degree of fluorescence polarization of the DPH probe in rat brain membranes treated with increasing concentrations of selected 3β-hydroxy-5-ene steroids. The values are expressed as per cents ± s.e.m. related to control samples with zero concentration of steroids. The degree of fluorescence polarization in control samples amounted 0.295 ± 0.005. Each value represents the mean of 3–4 determinations from two independent experiments

Compound	Concentration, mg/ml			
	0.000	0.030	0.100	0.300
Cholesterol hemisuccinate	100 ± 1.7	103 ± 0.3	107 ± 0.3[a]	110 ± 1.0[a]
Cholesterol	100 ± 0.3	101 ± 1.4	101 ± 1.4	105 ± 0.7
Cholesterol sulfate	100 ± 0.3	102 ± 3.0	101 ± 1.0	108 ± 7.0
DHEA	100 ± 2.7	99 ± 2.0	101 ± 0.6	96 ± 0.6
DHEA acetate	100 ± 2.7	96 ± 1.7	89 ± 2.3[b]	84 ± 1.7[a]
7α-OH-DHEA	100 ± 0.6	95 ± 0.6	97 ± 1.0	94 ± 0.6[b]
7α-OH-DHEA 3β-acetate	100 ± 1.7	99 ± 1.0	94 ± 1.0	99 ± 0.6
Pregnenolone	100 ± 0.7	103 ± 0.7	101 ± 1.3	88 ± 1.3[a]
Pregnenolone acetate	100 ± 1.7	102 ± 1.7	99 ± 0.6	94 ± 0.6[b]
7α-OH-cholesterol	100 ± 2.3	100 ± 1.7	98 ± 1.1	99 ± 1.2
7β-OH-cholesterol	100 ± 2.3	94 ± 0.8	96 ± 1.7	110 ± 0.3

Significance when compared to control samples: [a] $p < 0.01$, [b] $p < 0.05$.

DISCUSSION

The presence of 7α-hydroxylated DHEA in humans was reported for the first time in 1959 in urine and in a tumor tissue of a patient operated for adrenal carcinoma[27], and in urine of healthy men administered with large doses of DHEA (ref.[28]). Later on, it has been demonstrated to be present in urine of normal men[29].

7α-OH-DHEA originates from DHEA by action of a specific 7-hydroxylase as confirmed by several authors. The 7-hydroxylase activity due to a cytochrome P450-NADPH-dependent enzyme complex has been demonstrated first in rat liver[30], and later in many other mammalian tissues[6,31]. The occurrence of 7-hydroxylated DHEA in tissues and body fluids and the metabolism of the compound were recently reviewed[32]. 7α-Hydroxylation has been even found in various human fetal tissues and later on also in amniotic epithelium. Properties of the enzyme were studied more in detail in the rat liver[33,34] and in mouse tissues[16]. 7α-Hydroxylation of various 3β-hydroxy-C_{19} steroid substrates was demonstrated in normal and hyperplastic human prostate[35]. A high 7α-hydroxylase activity was also found in human and mouse skin[6,31].

The finding of a 7α-hydroxylase activity in malignant mammary tumor tissues, higher than in benign ones[36] prompted Skinner *et al.*[37] to investigate whether measurement of circulating 7α-OH-DHEA could be used as a marker for classification of the grade of the disease. However, no convincing results were obtained[37]. 7α-Hydroxylation was demonstrated in human skin and in adipose tissue[31,38] and in a number of murine tissues including brain, spleen, thymus, perianal and ventral skin, intestine, colon, coecum and muscle using a twin isotope technique[6]. The presence of 7-hydroxylated-5-ene steroids in the circulation and in tissues, and their biological effects which demonstrate antiglucocorticoid potencies, raise the question as to the molecular mechanism(s) of their action.

Using liver cytosols from adrenalectomized rats, we attempted to elucidate some aspects of the mechanism(s) which may be involved in the antiglucocorticoid effects of the 7α-hydroxylated derivatives of 3β-hydroxy-5-ene steroids. First, we could not find evidence for a cytosolic protein with specific binding capacity for [^{3}H]-7α-OH-DHEA. Thus we exclude a specific receptor-mediated mechanism of action. Second, a putative interference of 7α-OH-DHEA with glucocorticoid machinery was investigated. With liver cytosol from adrenalectomized rats, we obtained data proving that binding of [^{3}H]-DEX to the glucocorticoid receptor was efficiently decreased by glucocorticoids namely by DEX and corticosterone, and the RU486 antiglucocorticoid, but not by either DHEA or 7α-OH-DHEA.

However, DHEA, 7α-OH-DHEA, PREG and 7α-OH-PREG decreased the nuclear content of [^{3}H]-DEX-activated GRGC to an extent as large as the one brought about by identical concentrations of DEX. Furthermore, a significantly larger decrease induced by 7α-OH-DHEA than by 7α-OH-PREG indicates a molecular specificity of this effect. The fact that DHEA- and 7α-OH-DHEA-induced decreases of the nuclear [^{3}H]-DEX-

labelled GRGC were not statistically different may be explained by the reported 7α-hydroxylation of DHEA by isolated nuclei[16], but do not exclude a specific effect of DHEA itself. These findings prompted us to further investigate the possibility that, once translocated into the cell nucleus, 7α-hydroxy steroids may decrease the nuclear uptake of [^{3}H]-DEX-activated GRGC by impairing its binding to DNA. In order to study this possibility, the retention of crude or purified [^{3}H]-DEX-activated GRGC from rat liver cytosol on DNA-cellulose, DEAE-cellulose and hydroxyapatite columns was examined.

Through extensive experiments carried out under various conditions with this cell-free system, no significant effect of DHEA, PREG and of their 7α-hydroxylated derivatives was observed on the binding yields of the [^{3}H]-DEX-activated GRGC to DNA-cellulose, DEAE-cellulose or hydroxyapatite, thus excluding the possibility hypothesized above.

In contrast, we demonstrated that the fluidity of cell membranes was decreased by the steroids tested. Nevertheless, the effect is far from being decisive for their mode of action by potential influence on the accessibility of steroids to the intracellular receptors. Taken together, our results indicate that 7α-hydroxylated steroids prevent the nuclear retention (uptake) of activated GRGC, however large concentrations of DHEA and 7α-OH-DHEA are necessary for obtaining this effect. We have shown in mouse liver that 7α-hydroxylation of PREG and DHEA is mediated by mitochondria, microsomes and to a lesser extent by nuclei, and that the liver 7α-hydroxylation is the largest when compared with that of other tissues. Such yields infer that intracellular concentrations of 7α-hydroxylated metabolites may reach the levels required for prevention of the activated GRGC uptake by nuclei, and that autocrine or paracrine action of 7α-hydroxy steroids may be at stake. Nevertheless, the question arises as to the molecular mechanism of this cellular phenomenon. In the experiments with isolated nuclei, the effect due to the presence of 7α-OH-DHEA, DHEA and 7α-OH-PREG occurred solely when nuclei were preincubated with these steroids prior to the addition of the [^{3}H]-DEX-labelled GRGC. This prompted us to design cell-free experiments for testing the putative effect of 7α-hydroxy steroids on the binding of [^{3}H]-DEX-labelled GRGC to DNA-cellulose. The negative results of these investigations orient us toward other hypotheses and further studies.

As preincubation with 7α-hydroxy steroids is a necessary step for obtaining a decreased nuclear uptake of the GRGC (ref.[13]) and preincubation of mouse T cells with 7α-hydroxy steroid is a necessary to prevent DEX-induced apoptosis[7], two hypotheses may be assumed. First, 7α-hydroxy steroids may bind to the activated GRGC through a more complex mechanism leading to conformational modifications so that dimerization and/or nuclear translocation of the activated receptor might be impaired. Second, and not necessarily excluding of the first hypothesis, the 7α-hydroxy steroids may suf-

ficiently modify the nuclear membrane so that translocation of the activated GRGC is decreased. Both hypotheses are currently being investigated.

ABBREVIATIONS

List of less common symbols and abbreviations not explained throughout the text:

DEAE-cellulose	2-dimethylaminoethyl)cellulose
DMSO	dimethyl sulfoxide
DPH	1,6-diphenylhexa-1,3,5-triene
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)piperazine-1-(2-ethanesulfonic acid)
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffer saline
PMSF	phenylmethanesulfonyl fluoride
PVP	poly(vinylpyrrolidone)
TAPS	3-{[tris(hydroxymethyl)]amino}propane-1-sulfonic acid
Tris	2-amino-(2-hydroxymethyl)propane-1,3-diol

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