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Binding of the Anti-inflammatory Steroid Deflazacort to Glucocorticoid Receptors in Brain and Peripheral Tissues. *In Vivo* and *In Vitro* Studies

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Deflazacort (DFC) is a heterocyclic glucocorticoid with anti-inflammatory activity but with decreased side effects. In this study, we have evaluated the capacity of DFC and other glucocorticoids to reach the central nervous system (CNS) *in vivo* by measuring changes of [^{3}H]dexamethasone (DEX) binding to glucocorticoid receptors (GR) *in vitro*. GR occupation was effected by DEX in the cerebral cortex, hippocampus, pituitary, liver and thymus, with DFC showing a similar profile except for the cerebral cortex. In contrast, corticosterone weakly occupied GR in the thymus, pituitary and hippocampus and methyl-prednisolone was active only in peripheral tissues. Furthermore, IC_{50} for DEX *in vitro* amounted to 15–17 nM in the hippocampus and liver, whereas IC_{50} for the active metabolite 21-deacetyl-DFC (21-OH-DFC) was 4 times higher. 21-OH-DFC bound to type II and was absent from type I GR. When tested in equipotent doses based on IC_{50} analysis, DFC and DEX similarly induced *in vivo* ornithine decarboxylase activity in hippocampus and liver, although body weight loss after chronic treatment was significantly less for DFC. The results show that DFC distributes on the CNS similarly to DEX, induces ornithine decarboxylase activity but presents less intensive catabolic effects, making it suitable for use as an anti-inflammatory steroid during chronic therapeutic regimes.

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INTRODUCTION

Glucocorticoids are widely used for the clinical management of several human diseases. However, undesirable secondary effects complicate their long-term administration [1–3]. To preclude side effects, chemical modifications of the glucocorticoid molecule were devised aiming to preserve anti-inflammatory activity while reducing sodium retention, hyperglycemia, catabolism and adverse effects on bone metabolism [4]. In this connection, the synthetic steroid deflazacort (DFC) (11β-16β)-21-(acetoxyl)-11-hydroxy-2′-methyl-5′H-pregna-1,4-dieno(17,16d) oxazole-3,20-dione, was from 10 to 20 times as active as prednisolone in pharmacological tests of anti-inflammatory activity in rats [5]. However, hypercalciuria, negative calcium balance [6, 7], hyperglycemia with insulin resistance, [8, 9] and impairment of growth [10] were less pronounced with DFC than with prednisolone and other synthetic steroids.

An important property of natural and synthetic glucocorticoids is the feedback inhibition of the hypothalamic–pituitary–adrenal (HPA) axis [11]. Among the factors regulating HPA activity, glucocorticoid binding by neuroendocrine tissues plays an important role [12, 13]. However, a report using radiolabeled DFC injected into rats has shown its preferential uptake by liver and kidney with negligible concentration by whole brain [14]. Due to the importance of glucocorticoid receptors for negative feedback [12, 13, 15] and the sparse information on DFC action in central structures, we have compared, using *in vivo* and *in vitro* methods, the binding characteristics and agonist activity of DFC in the central nervous system (CNS) and peripheral tissues. In the present work, we

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report the affinity of DFC for glucocorticoid receptors (GR), its capacity for inducing the glucocorticoid-dependent enzyme ornithine decarboxylase (ODC) [16] and the effects of prolonged DFC administration on body and tissue weight.

EXPERIMENTAL

Experimental animals

Male Sprague–Dawley rats (~250 g) were kept in an air-conditioned room with controlled temperature (25°C) and lighting conditions (lights on from 0700 until 1900 h). Rats were used intact or were bilaterally adrenalectomized (ADX) under ether anesthesia 3–5 days before hormone treatment. ADX rats received 0.9% NaCl as drinking fluid.

Treatment of animals for in vivo receptor occupation and in vitro steroid receptor assays

As isotopically-labeled DFC is not commercially available, binding of [^{3}H]ligand to receptors of central and peripheral tissues was competed with DFC and binding inhibition compared to that of other steroids. This approach was employed in previous studies of DFC binding to receptors [17, 18]. For *in vivo* studies, ADX animals were given i.v. injections of vehicle (0.3 ml 20% ethanol in 0.9% NaCl) or 400 μg/kg of one of the following steroids: DFC, corticosterone (CORT), dexamethasone (DEX), methyl-prednisolone or β-hydroxy-prednisone. Sixty min after injection, animals were anesthetized with ether and perfused intracardially with 30 ml 10% dimethylsulfoxide (DMSO). This agent cryoprotects receptors during freezing and thawing [19]. Tissues (hippocampus, anterior pituitary, cerebral cortex, thymus and liver) were excised and stored at −70°C.

For *in vitro* studies, the hippocampus and liver were obtained from untreated ADX rats which were perfused with DMSO and stored at −70°C until assayed for steroid receptors.

GR assay

Previously reported methods were employed to determine binding to total glucocorticoid receptors in central and peripheral tissues or to type I and type II receptors in the hippocampus [20, 21]. For *in vivo* competition, frozen tissues obtained from ADX rats receiving vehicle or steroids (see above) were thawed and homogenized in 10 mM Tris pH 7.4, containing 1.5 mM EDTA, 2 mM mercaptoethanol, 10% glycerol and 20 mM sodium molybdate. Homogenates were centrifuged at 105,000 *g* for 60 min and aliquots of the cytosol were incubated with 10 nM [^{3}H]DEX in the presence or absence of a 1000-fold molar excess of nonradioactive steroid. Incubations lasted for 20 h at 4°C, at the end of which bound and free [^{3}H]DEX were separated on Sephadex LH-20 minicolumns. After subtraction of nonspecifically bound hormone, results of specific binding were expressed in fmol [^{3}H]DEX bound per mg protein.

For competition of [^{3}H]DEX binding *in vitro*, cytosol was prepared from hippocampus and liver of untreated ADX rats. Aliquots of the cytosol were labeled with 10 nM radioactive hormone and competed with a range (10–500 nM) of DEX, DFC or its active metabolite 21 desacetyl-DFC (21-OH-DFC) [14]. For non-specific binding, a 1000-fold excess of DEX was used. Results were plotted as percent binding of uncompeted incubations, and IC_{50} calculated [22].

For categorizing receptor subtypes, cytosol prepared from the hippocampus of ADX rats was incubated with 10 nM [^{3}H]CORT with or without 500 nM RU 26988 to block binding to type II receptors [21, 22]. Non-specific binding was determined after addition of a 1000-fold excess of unlabeled DEX. In the absence of RU 26988, [^{3}H]CORT bound to both type I and type II receptors, whereas radioactivity in the presence of RU 26988 represented binding to type I sites. Subtraction of this value from total binding yielded binding to type II sites [21]. Binding to type I and type II receptors was competed with 5–300 nM unlabeled DEX or 21-OH-DFC, and results were expressed as % [^{3}H]CORT bound vs log competitor concentration (M).

ODC assay

For this assay, animals were ADX 48 h before the experiment and divided into four groups. The 1st group (ADX only) received vehicle; the 2nd group was treated s.c. with 5 mg/kg DEX dissolved in 30% ethanol in 0.9% NaCl 6 h before killing. Maximal ODC induction is obtained under these conditions [23]. The 3rd group received a dose of DFC similar to that of DEX (5 mg/kg), whereas the 4th group received 20 mg/kg; animals were killed 6 h after injection. The higher dose of DFC was calculated to be equipotent to DEX, based on IC_{50} of the active metabolite 21-OH-DFC which was 4 times lower than IC_{50} for DEX in liver and hippocampus (see Results, Fig. 2).

Activity of ODC was measured according to Orti *et al.* [22]. Briefly, hippocampus and liver excised from the 4 groups of rats were homogenized in 50 mM KH_2PO_4 pH 7.2, 2 mM EDTA, 1 mM dithiothreitol, 30 mM NaN_3 and centrifuged at 12,000 *g*. The supernatant was incubated with 0.2 mM [^{14}C]ornithine and 0.1 mM pyridoxal phosphate during 60 min at 37°C. After addition of 40% trichloroacetic acid to stop the reaction, the [^{14}C]CO_2 released was trapped in hyamine hydroxide and counted. Results of ODC activity were expressed as pmol [^{14}C]CO_2 released/h/mg protein.

Treatment of animals for long-term effects of DFC and DEX

Intact rats were divided into three groups of 10 animals each. The 1st group received vehicle in the drinking water (0.2% ethanol); the 2nd group received 1 μg/ml of DEX, which resulted in an average dose

~100 μg/kg/day. The third group received 4 μg/ml DFC (450 μg/kg/day, because the DFC-treated rats drank slightly more than the DEX-treated rats). Three weeks later, animals were weighed and killed by decapitation. Body and tissue weights were recorded and expressed in g and in mg/g body wt.

RESULTS

Figure 1 shows [^{3}H]DEX binding to cytosolic glucocorticoid receptors in the cerebral cortex, hippocampus, anterior pituitary, liver and thymus after ADX rats received vehicle or different steroids. Receptor occupation was effected by DEX in the 5 tissues; DFC was as effective as DEX in the hippocampus, pituitary, liver and thymus, but less effective in the cerebral cortex. In contrast, CORT weakly occupied receptors in the thymus, pituitary and hippocampus, and methylprednisolone was inactive in the hippocampus and pituitary but active in the periphery (liver and thymus).

Further comparisons were made between DFC, its deacetylated metabolite (21-OH-DFC) and DEX. Determination of IC_{50} for DEX demonstrated that it amounted to 15–17 nM in the hippocampus and liver (Fig. 2). In both tissues, IC_{50} for 21-OH-DFC was 4 times higher than DEX. IC_{50} for DFC (the acetylated parent molecule) was similar to 21-OH-DFC in the liver, indicating the presence of cytosolic deacetylase, whereas the enzyme may be absent in cytosol of the hippocampus as suggested by the disparate increase in IC_{50} for DFC (870 nM).

The experiments reported in Figs 1 and 2 determined binding to total soluble receptors. To elucidate the participation of type I and type II sites in DFC action, we chose the hippocampus, considering its high content of both receptor forms [12, 13]. Figure 3 shows that although DEX preferentially occupied type II sites (upper graph), [^{3}H]CORT was also displaced from type I sites by DEX. In contrast, 21-OH-DFC exclusively displaced [^{3}H]CORT from type II receptors in the hippocampus (Fig. 3, lower graph).

As a marker of the glucocorticoid's biological activity, induction of ODC was studied in the hippocampus and liver (Fig. 4). In comparison to ADX rats, 5 mg/kg DEX markedly induced ODC in both tissues. *In vivo* administration of low dose DFC (5 mg/kg) significantly stimulated ODC in liver but not hippocampus, but 20 mg/kg (dose equipotent to DEX based on results of IC_{50}) was as effective as DEX in both tissues.

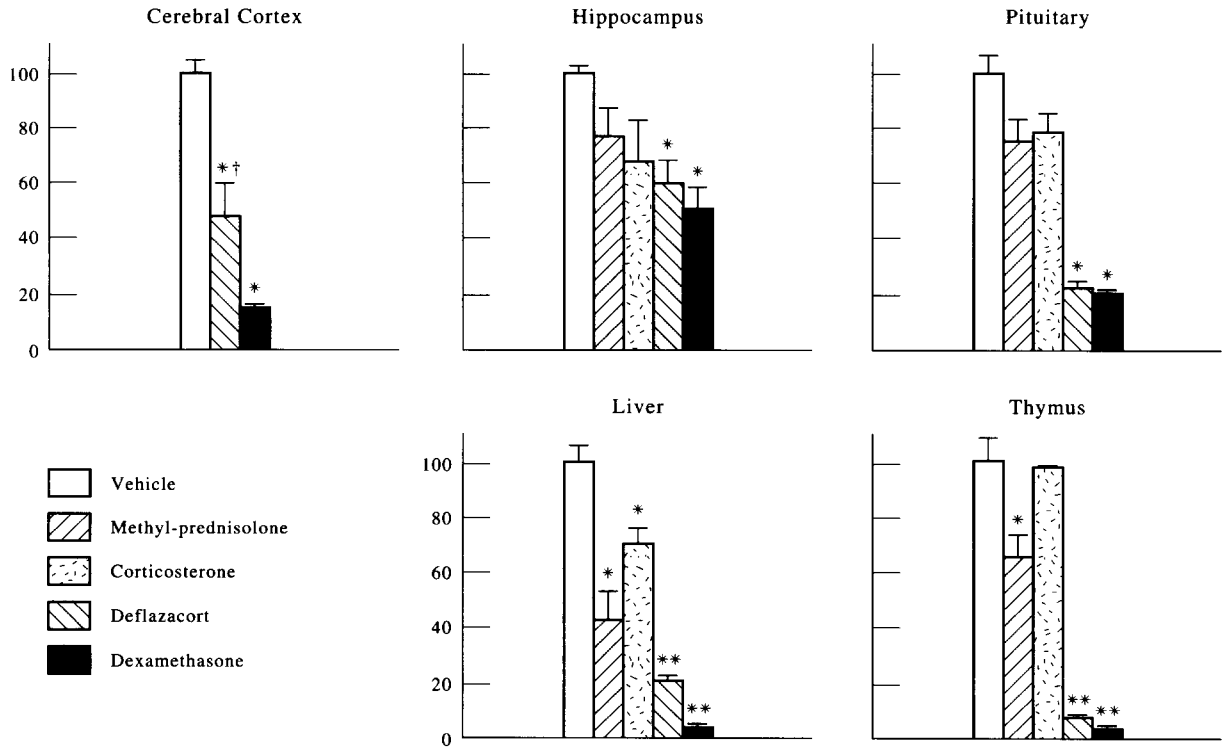
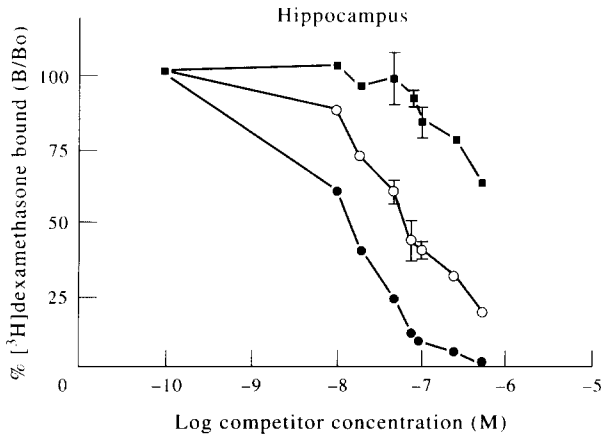


Fig. 1. Effect of a single i.v. administration of 400 μg/kg of nonradioactive steroids to ADX rats on binding of [^{3}H]DEX to cytosolic glucocorticoid receptors from central and peripheral tissues. Results are expressed as % binding (mean ± SE of 4–11 animals per group) respect to the control group injected with vehicle. Binding levels for controls (in fmol/mg protein) were: cerebral cortex 225 ± 11; hippocampus 247 ± 75; pituitary 103.2 ± 77; liver 1092 ± 52.4 and thymus 492 ± 43.2. *$P < 0.05$, **$P < 0.01$ vs control group; †$P < 0.05$ vs DEX-treated group, by one way ANOVA followed by Scheffe's F test.



IC_{50} (nM)	Hippocampus	Liver
● Dexamethasone	15.91 ± 0.92	17.75 ± 0.75
○ 21-OH-DFC	73.53 ± 5.89	77.86 ± 2.19
■ DFC	870.25 ± 125.41	66.14 ± 3.02

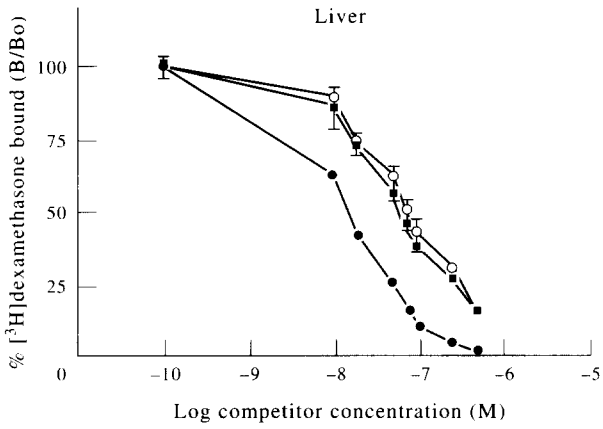


Fig. 2. Competition of [^{3}H]DEX binding by *in vitro* addition of DFC and its deacetylated metabolite (21-OH-DFC). Cytosol from hippocampus (upper graph) or liver (lower graph) was incubated with 10 nM radioactive ligand in the absence or presence of 10^{-5} to 10^{-9} M competitor for 20 h at 0–4°C. Values for IC_{50} are shown in the middle part of the figure. IC_{50} was 4 times higher for 21-OH-DFC and DFC in liver and for 21-OH-DFC in hippocampus with respect to DEX, whereas in the latter tissue DFC showed very weak activity. Binding levels for uncompeted samples (in fmol/mg protein) were: hippocampus 322.1 ± 18.8 ($n = 6$); liver 881.3 ± 19 ($n = 6$).

Lastly, we compared the effects on body and tissue weight of DEX and DFC given for 3 weeks in the drinking water of adrenal-intact rats. Based on IC_{50} data, we chose a dose of DFC (4 μg/ml) equipotent to DEX (1 μg/ml). Figure 5 demonstrates that DEX was more catabolic than DFC regarding body weight. Table 1 shows that the adrenal, thymus, liver and spleen weights were similarly reduced by both compounds. After DFC and DEX treatment, relative tissue weight (mg/g body wt, Table 1) was significantly increased for the testis and kidney, reflecting a partial resistance by these organs to the catabolic effects of the synthetic glucocorticoids.

DISCUSSION

Previous studies have demonstrated that 21-OH-DFC is the active metabolite of DFC circulating in plasma and present in tissues after systemic or oral administration of the acetylated molecule to humans and experimental animals [14, 17]. Based on these findings, we assumed that the compound that reached central and peripheral GCR after i.v. injection of DFC was the 21-OH-derivative rather than DFC itself. Although tested in single doses, occupation of receptors by DEX and 21-OH-DFC seemed equivalent, except for the cerebral cortex, in which DEX occupation was

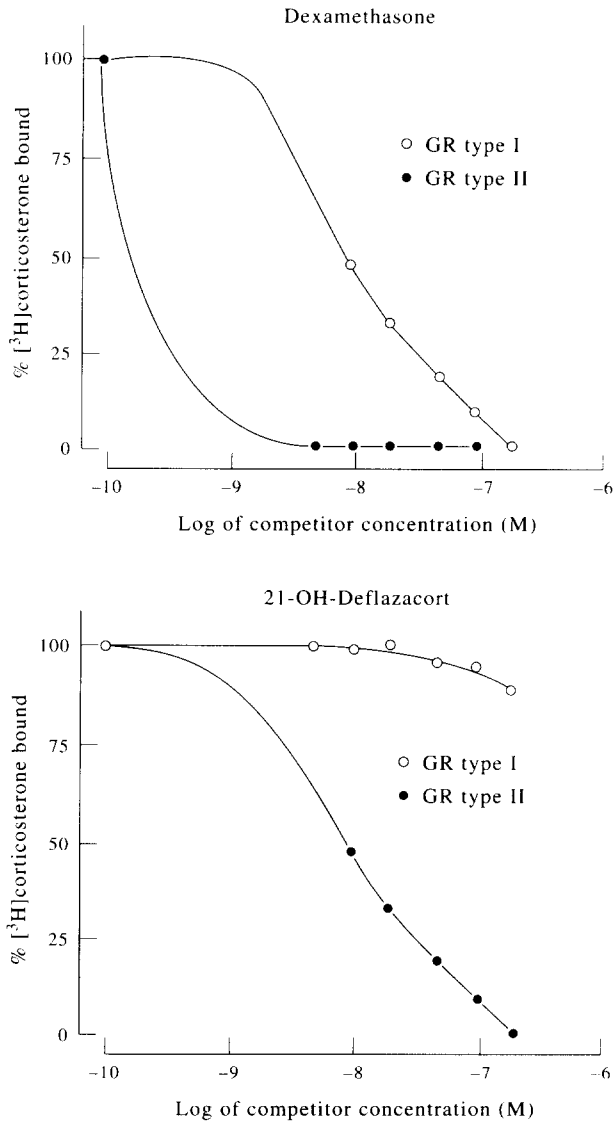


Fig. 3. Competition curves of [^{3}H]CORT (10 nM) binding in cytosol of hippocampus of ADX rats with 5–300 nM non-radioactive DEX or 21-OH-DFC. Binding to type I and type II corticosteroid receptors was determined by a subtraction method based on the presence or absence of 0.5 μM RU 26988. The figure shows that [^{3}H]CORT was displaced from both sites by DEX, whereas 21-OH-DFC was active on type II receptors only. Binding levels for uncompeted samples (in fmol/mg protein) were: type I sites 45.2 ± 8.2 ($n = 3$); type II sites 128.6 ± 9.5 ($n = 3$).

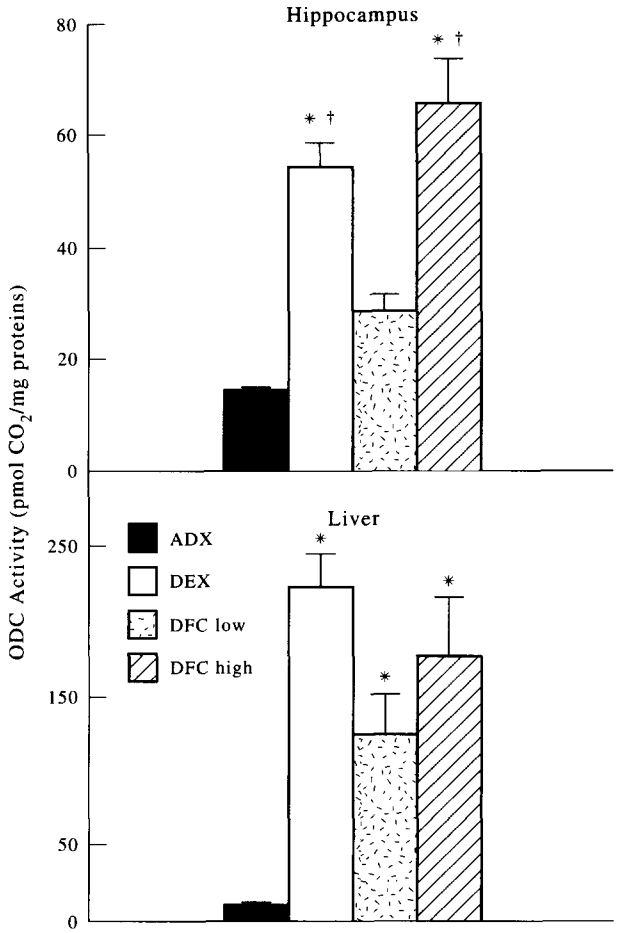


Fig. 4. Activity of ODC in hippocampus and liver from ADX rats receiving vehicle (shaded columns), 5 mg/kg DEX (open columns), 5 mg/kg DFC (DFC Low, stippled columns) or 20 mg/kg DFC (DFC High, hatched columns) 6 h before killing. The response of ODC to steroid treatment was similar in both tissues. Values represent the mean ± SE of 5–6 animals per group. Enzyme activity (pmol/h/mg protein) for control hippocampus was 14.4 ± 0.7; control liver 10.1 ± 1.2. *$P < 0.05$ vs ADX group; †$P < 0.05$ vs DFC Low, by one way ANOVA followed by Scheffe's F test.

significantly greater. The low effectiveness of CORT may be due to (1) plasma sequestration by transcortin, which reduces the biological activity of the hormone [24] and (2) low affinity towards binding sites labeled with [^{3}H]DEX [21]. Similar explanations could account for the behavior of methyl-prednisolone, although it showed a higher degree of occupation of liver and thymus rather than pituitary and central receptors. It is likely that both 21-OH-DFC and DEX were preferentially taken up by target tissues because they were not bound to steroid binding plasma proteins such as transcortin [14].

Second, we have observed that affinity of 21-OH-DFC for glucocorticoid receptors is lower than DEX, and that in the liver but not hippocampus, comparable IC_{50}s were found for DFC and 21-OH-DFC. It is possible that DFC was metabolized to its 21-OH derivative mostly in the liver, but it needed full deacylation by serum enzymes before entering the CNS. Our data confirm the *in vitro* work of Assandri *et al.* [14] that affinity of 21-OH-DFC for GCR is lower than DEX in cytosol of peripheral target organs. Luzzani and Glasser [17] and Luzzani *et al.* [18] found that 21-OH-DFC has higher affinity for GCR in the thymus, kidney and liver than DFC; we partly confirm this finding in the hippocampus but not liver.

Third, using the hippocampus as a representative area enriched in type I and type II receptors, we demonstrated that 21-OH-DFC binds exclusively to type II and is absent from type I receptors, whereas as in previous work, DEX bound in cytosol to both receptor types [25]. This may represent a therapeutic advantage for DFC because side effects such as hypertension and sodium retention are probably mediated via interaction of glucocorticoids with type I receptors [26]. The preliminary results of Buniva *et al.* [27] also suggested a weak effect of DFC on electrolyte balance.

Fourth, when tested in equipotent doses based on IC_{50} analysis, DFC and DEX similarly induced ODC in central and peripheral tissues, indicating its glucocorticoid activity. Fifth, long-term treatment with DFC in a dose 4 times higher than DEX had less severe catabolic effects on body weight, although the same dose induced ODC as strongly as DEX. Sixth, adrenal atrophy appeared after prolonged DFC and DEX therapy. This finding suggests a strong negative feedback mechanism at neuroendocrine tissues probably

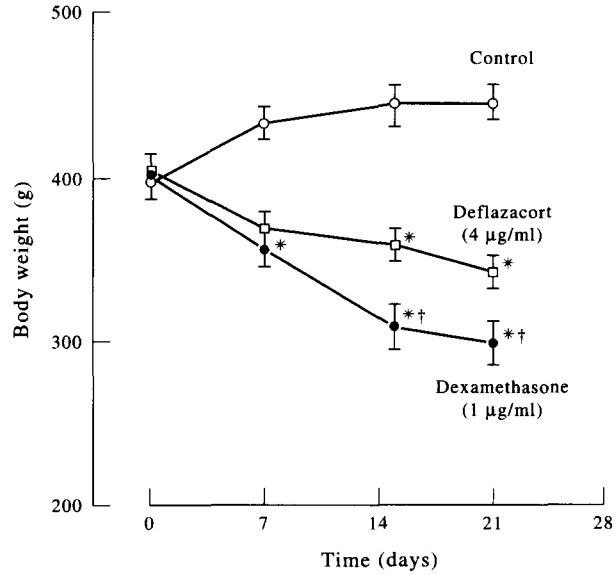


Fig. 5. Effects of DEX and DFC on body weight. Intact animals were given vehicle, 1 μg/ml DEX or 4 μg/ml DFC in the drinking water for 3 weeks. Rats were weighed before treatment started and every 7 days. Values are the mean ± SE of 10–12 animals per group. Significant effects on body weight loss were found for both DEX and DFC after the 1st week (*$P < 0.05$). DEX showed a significantly higher catabolic effect than DFC after 2 and 3 weeks of treatment (†$P < 0.05$), by two way ANOVA followed by Duncan's multiple range test.

Table 1. Effects of DEX and DFC on tissue weight

	Liver	Spleen	Thymus	Testis	Adrenal glands	Kidneys
			Organ tissue weight (g)			
Control	15.68 ± 0.80	1.00 ± 0.09	0.68 ± 0.07	1.77 ± 0.12	0.021 ± 0.004	1.52 ± 0.07
Dexamethasone	11.18 ± 0.90*	0.54 ± 0.07*	0.45 ± 0.05*	1.78 ± 0.09	0.014 ± 0.003*	1.41 ± 0.08
Deflazacort	12.24 ± 0.56*	0.60 ± 0.04*	0.42 ± 0.03*	1.84 ± 0.11	0.014 ± 0.002*	1.50 ± 0.08
			Organ tissue/body wt (mg/g)			
Control	35.13 ± 1.57	2.26 ± 0.23	1.50 ± 0.20	3.95 ± 0.26	0.045 ± 0.007	3.42 ± 0.15
Dexamethasone	36.97 ± 1.89	1.76 ± 0.15	1.49 ± 0.14	6.02 ± 0.24*	0.051 ± 0.018	4.73 ± 0.12*
Deflazacort	35.96 ± 1.50	1.77 ± 0.12	1.22 ± 0.06	5.38 ± 0.27*	0.042 ± 0.006	4.18 ± 0.28*

Treatment of animals was described in the legend to Fig. 5. Values represent the mean ± SEM (n = 10–12 animals per group). *$P < 0.05$ vs control group by one way ANOVA followed by Scheffe F Test.

mediated by type II receptors [28], considering the high level of receptor occupation in the hippocampus and pituitary achieved after systemic administration of DFC and DEX. Two reports [27, 29] suggested that inhibition of plasma cortisol was lower with DFC than with prednisolone. However, this assumption needs corroboration with specific tests of inhibition and stimulation of the activity of the HPA axis.

From the point of view of the clinical utility, the analyzed parameters indicate that DFC is less catabolic than other potent glucocorticoids, with preservation of agonist activity. By extrapolation, it supports expectations of its usefulness in children, since it will inhibit growth to a lesser extent than other steroid analogs [7, 30]. Its low localization in the cerebral cortex may be therapeutically desirable for systemic diseases, in view of the fact that glucocorticoids in supraphysiological doses have deleterious and inhibitory effects upon the CNS [31].

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