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Research Article

Glucocorticoid Receptors in Human Synovial Tissue and Relative Receptor Affinities of Glucocorticoid-21-Esters

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A dexamethasone binding protein was detected in cytosol of 11 human synovial tissues from patients with chronic polyarthritis. The apparent dissociation constant (K_D) ranged from 3.3 to 17.1 (mean, 7.0 ± 4.3) nmol/liter, and the receptor concentration (R_o) from 17 to 65 (mean, 42 + 15) fmol/mg protein. Results of competition assays with an excess of unlabeled aldosterone, estradiol, pregnenolone, and testosterone confirmed that the binding protein had characteristics of a glucocorticoid receptor. With the use of diisopropylfluorophosphate (DFP) for esterase inhibition, and considering the purity of the starting material and the hydrolysis products, we could determine the relative receptor affinities of glucocorticoid-21-esters. In contrast to the high affinity of the glucocorticoid-17-ester examined, esterification in position 21 abolishes binding affinities. Glucocorticoid-21-esters are true prodrugs for which the glucocorticoid action is caused only by the corresponding glucocorticoid alcohol.

KEY WORDS: glucocorticoid receptors; synovial tissue; relative receptor affinity; glucocorticoid-21-ester; diisopropylfluorophosphate; esterase inhibition.

INTRODUCTION

Glucocorticoids play an important role in the therapy of inflammatory processes. Since 1951 (1) they have been used intraarticularly to reduce symptoms such as swelling, redness, pain, and high joint temperature. Hollander *et al.* (2) recognized that local application of glucocorticoid solutions had only short effects and started to use water-insoluble glucocorticoid esters as crystalline suspensions. Local application is successful only when the inflamed tissue contains glucocorticoid receptors (3,4). To our knowledge the presence of glucocorticoid receptors in synovial tissue has not yet been demonstrated. The extent of pharmacological effects depends on the receptor affinity of the applied glucocorticoid. 17-Esters possess higher affinities than the corresponding alcohols (5–7). During the determination of relative receptor affinities (RRA) the glucocorticoid-17-esters and alcohols (8,9) are stable in incubation medium, in contrast to the 21-esters (9–11). The extent of ester cleavage of the unstable 21-esters depends on the side-chain length (9,10). RRAs given in Ref. 6 represent only upper limits for 21-esters since the applied esterase inhibitor did not guarantee total inhibition of ester cleavage.

Because of the importance of glucocorticoid-21-esters in the management of articular diseases, it is interesting to investigate the occurrence of glucocorticoid receptors in human synovial tissue as well as to determine the receptor affinities of glucocorticoid-21-esters.

MATERIALS AND METHODS

DFP (diisopropylfluorophosphate) was purchased from Janssen Chimica (Nettetal, FRG), and ^{3}H-dexamethasone and ^{3}H-triamcinolone acetonide were obtained from NEN (Boston). Radiochemical purity was demonstrated by thin-layer chromatography (TLC). Scintillation counting was done with a Tricarb 300 C from Packard Instruments (Zürich, Switzerland) using Quickszint 212 from Zinsser (Frankfurt/Main, FRG).

High-Performance Liquid Chromatographic (HPLC) System. The HPLC system was as follows: solvent delivery system SP 8700 and integrator minigrator (Spectra Physics, Darmstadt, FRG); injection loop (self-made); and aerograph UV detector, 254 nm (Varian Instruments, Calif.).

Handling and Collecting of Tissue Samples. Immediately after resection, synovial tissues were frozen in a mixture of acetone and dry ice and stored for 2 weeks at most. Then they were either analyzed immediately or stored in liquid nitrogen for examination within 2 months. Eleven synovial tissues were taken from patients with chronic polyarthritis who had to undergo synovectomy.

Preparation of cytosol was performed as described by Hochhaus *et al.* (12) with the exception that the buffer contained no EDTA, but 5 mmol/liter DFP. The protein concentration of cytosol was measured using the method of Lowry *et al.* (13).

Assay for Determination of R_o *and* K_D. We followed the method of Toft und Gorski (14); cytosol was incubated

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Table I. Chromatographic Conditions for Determination of Hydrolysis Products[a]

Mobile phase (v:v)				Separation of			Internal standard
H_2O	CH_3CN	CH_3OH	CH_3COOH				
420		530	5	MP	MPA		Pred
700	300		20	Pred	Pred-HS		Dexa
700	250		20	Dexa	Dexa-P		Pred
700	240	180	2	Tr	TMA	TDA	MP
200	50	400	20	Pred	P-17-E	Prednic	Bud

[a] MPA, methylprednisolone-21-acetate; Dexa-P, dexamethasone-21-phosphate; Pred-HS, prednisolone-21-hemisuccinate; Tr, triamcinolone; TDA, triamcinolone-16,21-diacetate; TMA, triamcinolone-21-acetate; Bud, budesonide; P-17-E, prednisolone-17-ethylcarbonate; Prednic, prednicarbate.

with different concentrations of ^{3}H-dexamethasone alone or in the presence of an excess of unlabeled dexamethasone. R_o and k_D were calculated by the method of Scatchard (15). For details see Hochhaus *et al.* (12).

Quantification of Hydrolysis Products and Purity of Glucocorticoid Esters. Hydrolysis products were measured by HPLC on RP18-columns with an internal standard. Chromatographic conditions are listed in Table I. The extraction procedure is described by Derendorf *et al.* (16).

The purity of esters was determined on RP18-columns with external standards at the following concentrations: esters, 0.1 mg/ml; and alcohols, 0.001, 0.002, and 0.003 mg/ml.

Determination of RRAs of Glucocorticoid-21-Esters. RRAs of glucocorticoid-21-esters and the corresponding alcohols were determined by competition assays. Cytosol was incubated for 6 hr at 20°C with ^{3}H-triamcinolone acetonide and different concentrations of unlabeled competitors. The competitor concentration necessary to displace 50% ^{3}H-triamcinolone acetonide ($C_{x,50}$) was calculated relative to the reference substance dexamethasone (= 100) as follows:

$$RRA_x = 100 \cdot C_{Dexa,50}/C_{x,50}$$

At least two experiments were carried out for each competitor, with up to 10 different concentrations. Correlation coefficients for linearized binding curves averaged 0.993 (N = 43). [For details see Rohdewald *et al.* (8).]

RESULTS

R_o and K_D of Glucocorticoid Receptors in Synovial Tissue

A typical plot for total, specific, and nonspecific binding is shown in Fig. 1. Scatchard analysis of specific binding (Fig. 2) gave straight lines, indicating a single class of high-specific, low-capacity binding sites. R_o and K_D were calculated to range from 17 to 65 (mean, 42 ± 15) fmol/mg protein and 3.3 to 17.1 (mean, 7.0 ± 4.3) nmol/liter, respectively.

To demonstrate glucocorticoid specifity, competition assays using an 100-fold excess of unlabeled triamcinolone acetonide, dexamethasone, hydrocortisone, estradiol, pregnenolone, aldosterone, and testosterone were performed. Only glucocorticoids were able to displace labeled triamcinolone acetonide from the receptor, while the other steroids had only little effect (Table II).

Hydrolysis of Glucocorticoid Esters

During 6-hr incubations at 20°C in human synovial cy-

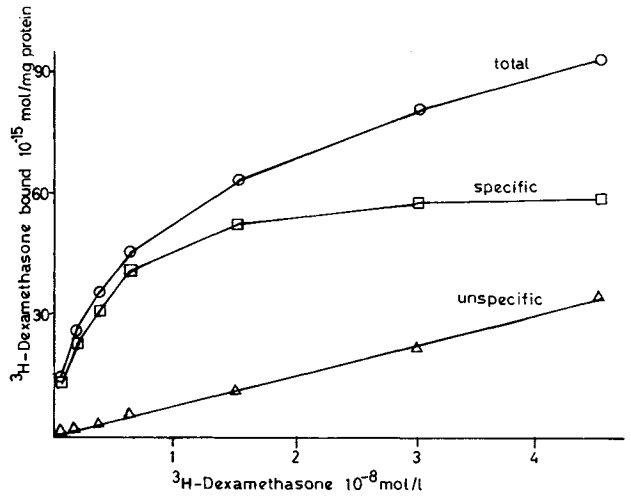

Fig. 1. Total, specific, and nonspecific binding of dexamethasone to inflamed human synovial tissue.

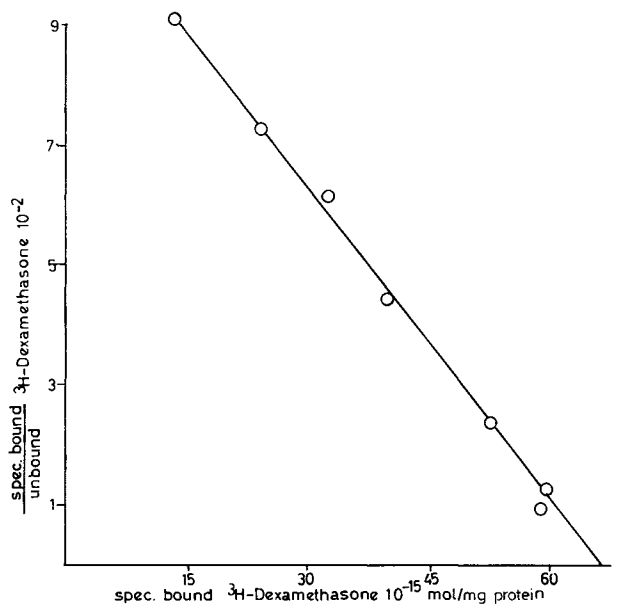

Fig. 2. Scatchard plot calculated from the values for specific bound dexamethasone from Fig. 1.

Table II. Inhibition of ^{3}H-Triamcinolone Acetonide Binding in Cytosol of Four Different Synovial Tissues by a 100-fold Excess of Various Unlabeled Steroids

Unlabeled steroid	Percentage displacement			
Triamcinolone acetonide	103	101	96	101
Dexamethasone	100	100	100	100
Hydrocortisone	75	68	82	73
Testosterone	3	10	10	9
Estradiol	4	11	6	6
Pregnenolone	1	4	2	1
Aldosterone	11	8	6	7

tosol, DFP at $2.7 \cdot 10^{-4}$ mol/liter decreased the hydrolysis of methylprednisolone-21-acetate from 71.3 to 4.10% and of prednisolone-21-hemisuccinate from 14.8 to 3.4%. DFP had no significant effect on the hydrolysis of dexamethasone-21-phosphate (Table III).

On the basis of data from Distelhorst and Miesfeld (17), we examined hydrolysis of three additional glucocorticoid esters in synovial cytosol containing $5 \cdot 10^{-3}$ mol/liter DFP. Ester cleavage during 6-hr incubations was always below 2% (Table III).

Table III. Dependence on Ester Cleavage in Human Synovial Cytosol in DFP Concentration During 6-hr Incubations at 20°C

Ester	DFP concentration (mol/liter)	Ester cleavage (%)
Prednisolone-21-hemisuccinate	0	14.8
	$2.7 \cdot 10^{-4}$	3.4
Dexamethasone-21-phosphate	0	43.0
	$2.7 \cdot 10^{-4}$	26.9
Methylprednisolone-21-acetate	0	71.3
	$2.7 \cdot 10^{-4}$	4.1
	$1.35 \cdot 10^{-4}$	7.1
	$6.68 \cdot 10^{-5}$	11.5
Triamcinolone-16, 21-diacetate	$5 \cdot 10^{-3}$	1.9
Prednicarbate	$5 \cdot 10^{-3}$	1.5
Prednisolone-17-ethylcarbonate	$5 \cdot 10^{-3}$	2.0

Free Glucocorticoid Alcohol Content in Glucocorticoid Esters

Quantification of glucocorticoid alcohols in glucocorticoid esters was important because the presence of glucocorticoid alcohol would falsify the determination of the RRA of its respective esters. Free glucocorticoid alcohol concentrations ranged from 0 to 15.2% in different esters (Table IV).

Relative Receptor Affinities

To determine values for receptor affinities of glucocorticoid ester (RRA_E), data evaluated in competition assays (RRA_U) must be corrected by subtraction of the receptor affinity of glucocorticoid alcohol produced by hydrolysis in the incubation medium (RRA_H) as well free glucocorticoid alcohols in the examined esters (RRA_v). The receptor affinity of 21-esters can be calculated using the following formula:

$$RRR_E = RRA_U - RRA_H - RRA_v$$

with $RRA_H = 0.02\ RRA_{alcohol}$ (taking into account a maximal hydrolysis of 2%) $RRA_v = 0.01y\ RRA_{alcohol}$, and y = percentage alcohol contained in glucocorticoid ester.

With these criteria, none of the glucocorticoid-21-esters had a measurable receptor affinity (Table IV), in contrast to the examined glucocorticoid-17-ester. Therefore, glucocorticoid-21-esters are true prodrugs, and their glucocorticoid actions are caused only by the corresponding alcohols.

DISCUSSION

Glucocorticoid Receptors

The glucocorticoid receptors in synovia are comparable to those found in other human tissues (Table V). There are no clear differences for K_D, which range from 4 to 17 nmol/liter. On the basis of receptor concentrations, tissues can be divided into two groups with either 49–90 fmol/mg protein (e.g., synovia) or three- to fourfold higher levels (hypothalamus, pituitary gland, and lymphoepithelial thymus gland).

Relative Receptor Affinities

In Table VI relative receptor affinities in synovia are

Table IV. Calculated Values for RRA of Glucocorticoid Esters (RRA_E) Taking into Consideration Receptor Affinity Caused by Steroid Alcohol Contaminants (RRA_v) or Resulting from Ester Hydrolysis During Incubation (RRA_H)[a]

Glucocorticoid ester	X	RRA_U	RRA_H	RRA_v	RRA_E
Dexamethasone-21-acetate	0	2	2		0
Triamcinolone hexacetonide	0	1	4		0
Triamcinolone diacetate					0
Triamcinolone-21-acetate					0
Methylprednisolone-21-acetate	1.0	1	0.54	0.27	0.19
Methylprednisolone-21-hemisuccinate	15.2	5	0.54	4.2	0.26
Prednisolone-21-acetate	0.5	0.32	0.28	0.07	0
Prednisolone-21-hemisuccinate	0.5	0.65	0.28	0.07	0.30
Prednicarbate	2.0	3	1.48	1.48	0.04
Hydrocortisone-21-acetate	0	0.30	0.22		0.08

[a] RRA_U = values from competition assays; X = % steroid alcohol contaminant in the glucocorticoid esters.

compared with those in human lung tissue (6). Values for glucocorticoid alcohols mostly coincide. Our data for triamcinolone are based on a broader experimental basis for calculation of $C_{x,50}$ than in Ref. 6. The reason for the different values of methylprednisolone is not known.

In a previous report (6) glucocorticoid alcohols had higher affinities than their corresponding 21-esters. However, the utilized esterase inhibitor did not assure complete inhibition of ester cleavage (6), and quantification of hydrolysis products was not performed. In our experiments both values were determined so that exact data for receptor affinities of 21-esters could be evaluated.

In agreement with our data, Ponec *et al.* (5) show high affinities for glucocorticoid-17-esters. In addition, they reported a dependence of the receptor affinity on the length of the side chain. In contradiction to our results, experiments resulted in receptor affinities for 21-esters that also were dependent on the length of the side chain. Esters with longer side chains (up to five carbon atoms) had higher affinities than esters with short side chains. Variations in incubation conditions may be the reason for these differences. Our calculations take into account the minimal hydrolysis in the presence of DFP for esterase inhibition. Ponec *et al.* (5) incubated glucocorticoid esters in cytosol of human keratinocytes for 3 hr at 0°C neither using esterase inhibitors nor controlling potential hydrolysis, although metabolism can take place even at low temperatures. Bonne and Raynaud (24) showed that 60% of dihydrotestosterone was metabolized, regardless of temperature, in prostata cytosol during 6-hr incubations at 0, 15, and 25°C.

Further, O'Neill and Carless (9) reported that hydrolysis of hydrocortisone-21-esters in skin homogenates of guinea pigs and hamsters depended on the side-chain length. Hydrocortisone-valerate and -butyrate showed greater cleavage during 30 min of incubation compared to hydrocortisone-acetate, -propionate, -octanoate, and -tetradecanoate. Wright *et al.* (10) used synovial cytosol for the determination of hydrolysis of cortisone-21-esters. Esters with a side-chain length of 4–10 carbon atoms were less stable than the acetate ester and esters with side chains containing more than 10 carbon atoms. The most unstable ester was cortisone-hexanoate.

Summarizing our results and the studies by O'Neill and Carless (9) and Wright *et al.* (10), the data published by Ponec *et al.* (5) may be interpreted as follows: During 3-hr incubation at 0°C, part of the glucocorticoid esters used in competition assays might have been hydrolyzed. As mentioned above, esters with longer side chains are cleaved faster than those with shorter side chains, which leads to apparent higher affinities of the esters with long side chains.

Table V. Comparison of Glucocorticoid Receptor Content and Apparent Dissociation Constant in Different Human Tissues

Tissue	N	Steroid	R_0 (fmol/mg protein)	K_D (nmol/liter)	Ref. No.
Synovial tissue	11	Dexa	42	7	
Lung	12	Dexa	63	15	6
Liver	7	Dexa	68	17	18
Skeleton muscle	14	Dexa	53	6	19
Epidermis	1	Dexa	95	16	20
Thymus gland (neoplastic)					
(a) epithelial	1	Dexa	96	4	21
(b) lymphoepithelial	4	TRA	278	7	22
Brain					
(a) pituitary gland	5	Dexa	300	7	23
(b) hypothalamus	5	Dexa	270	8	23

Table VI. Relative Receptor Affinities of Glucocorticoids Determined in Human Lung Cytosol (6) and in Cytosol of Human Synovial Tissue

Glucocorticoid	Relative receptor affinity	
	Synovial tissue	Lung
Dexamethasone	100	100
Dexamethasone-21-acetate	0	<18
Triamcinolone acetonide	255	233
Triamcinolone hexacetonide	0	
Triamcinolone-16,21-diacetate	0	
Triamcinolone-21-acetate	0	
Triamcinolone	3	9
Methylprednisolone	27	42
Methylprednisolone-21-acetate	0.19	<10
Methylprednisolone-21-hemisuccinate	0.26	<10
Hydrocortisone	11	9
Hydrocortisone-21-acetate	0.08	
Prednisolone	14	16
Prednisolone-21-acetate	0	
Prednisolone-21-hemisuccinate	0.30	
Prednisolone-17-ethylcarbonate	74	
Prednicarbate	0.04	

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