CORTISOL-BINDING PROTEIN IN THE CYTOSOL OF RAT CARRAGEENIN GRANULOMA

Yasuko KOSHIHARA, Micko YAMAGISHI, Sei-itsu MUROTA
and Susumu TSURUFUJI*

*Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku,
Tokyo-173, and *Department of Biochemistry, Faculty of Pharmaceutical Sciences,
Tohoku University, Sendai-980, Japan*

Accepted February 21, 1975

Abstract—Cortisol-binding protein was prepared and partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose column chromatography from 105,000 g supernatant fraction of cytoplasm in rat carrageenin granuloma, which is assumed to be one of the most appropriate experimental models of inflammation. The cortisol-binding protein in the inflammatory tissue, although similar to transcortine, was not transcortine itself. The binding protein was eluted at 0.12 M NaCl by DEAE-cellulose column chromatography with a shallow salt gradient. Sedimentation constant and dissociation constant of the binding protein were 4–5 S and 1.0×10^{-9} M, respectively. Optimum pH for binding to cortisol was 8.0. Binding ability of the binding protein to cortisol was very sensitive to pronase E and trypsin but resistant to RNase. Specificity of the protein for binding other steroids revealed that 17β -estradiol did not bind to the protein, while androstenedione and testosterone had one sixth as much affinity to the binding protein as that of cortisol. There was good a correlation between the amount of the binding protein in the inflammatory tissue and anti-inflammatory effect of cortisol. Namely, the maximum cortisol-binding ability was seen on a 5 day old granuloma which is the so called 'steroid sensitive stage'. Thereafter, the binding ability decreased with the increasing stage of granuloma.

Carrageenin granuloma is widely known to be one of the most appropriate experimental models of inflammation, and provides a useful tool for evaluating anti-inflammatory activity of drugs. Betamethasone, is a typical gluco-corticoid with strong anti-inflammatory activity in inhibiting formation of carrageenin granuloma and reducing pre-existing carrageenin granuloma. The granuloma pouch wall has been reported to become maximum in wet weight at day 5, while accumulation of pouch fluid takes place mainly in the next few days. When similar steroidal drugs are applied to the preformed granuloma daily for 4 days starting from the 5th day of the granuloma formation, the weight of pouch wall is reduced markedly. Non-steroidal anti-inflammatory drugs failed to reduce the pre-existing granuloma (1).

In the case of anti-inflammation by such steroidal drugs certain macromolecules are considered to play an important role on transport of the drugs. For example, they may be considered just like cortisol-binding protein located in cytoplasm and nuclei of liver (2–5). In this report, we attempted preparation of the cortisol-binding protein from cytoplasm of the 8 day old granuloma which is reportedly the maximum in size containing pouch wall and fluid (1) and purified the cortisol-binding protein by DEAE-cellulose

column chromatography with a shallow salt gradient. These preparations were examined both *in vivo* and *in vitro* labeling with ^3H -cortisol.

Also, the amount of cortisol-binding proteins in the granuloma cytosol and dissociation constants were investigated in various stages of granuloma formation, and correlation between the amount of the binding protein and effectiveness of the drug is discussed.

MATERIALS AND METHODS

Materials

Carrageenin granulomas were induced in male rats of Donryu strain and Sprague-Dawley strain (150 ± 10 g body weight) by the method of Fukuhara and Tsurufuji (1) except that Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.) was used instead of TS36 carrageenin. Four ml of 2% (W/V) carrageenin in saline was injected into the air sac (8 ml of volume) which had formed previously on the dorsum of rat, into which air had been injected subcutaneously.

Hydrocortisone-1,2,6,7- ^3H (spec. act. 82.7 Ci/mmole) was purchased from the New England Nuclear Co., Boston, U.S.A.. Hydrocortisone, 17 β -estradiol and collagenase (from *Cl. histolyticum*, 140 unit/mg) from Sigma Chemical Co., U.S.A., testosterone and androstene-3,17-dione from E. Merck Co., Germany, pronase E from Kaken Kagaku Co., Japan, trypsin from Difco laboratories, U.S.A., and chymotrypsin and ribonuclease from BDH Chemicals LTD., England, respectively.

Preparation of ^3H -cortisol labeled cytosol protein in vitro

Cortisol-binding protein fraction was prepared from cytosol of 8 day old carrageenin granuloma. Granulation tissue was minced and a 30% homogenate (W/V) was prepared with an ice cold TKMM buffer (0.02 M tris-HCl containing 0.015 M KCl, 0.003 M MgCl_2 and 0.01 M 2-mercaptoethanol, pH 7.4) which had been used in preparation of cortisol-binding protein from rat liver cytosol (3). All procedures were performed at 0–4°C unless otherwise mentioned. Homogenizing condition by VirTis homogenizer was set at 10,000 rpm for 10 sec in two times so that the nuclei remained intact. Granuloma cytosol fraction was prepared according to the method of differential centrifugation described by Beato *et al.* (2). A final supernatant fraction, at 105,000 g for 60 min, which contained cortisol-binding protein, was used for labeling ^3H -cortisol. Labeling the cytosol fraction with ^3H -cortisol was performed by incubating the mixture at 0°C for 60 min, unless otherwise described.

Preparation of ^3H -cortisol labeled cytosol protein in vivo

Twenty-five μCi of ^3H -cortisol with non-radioactive carrier cortisol dissolved in physiological saline were intravenously injected into a rat. After 45 min, the animal was sacrificed and a cytosol fraction containing tritiated cortisol-binding protein was prepared by the same manner as mentioned above.

Equilibrium dialysis

One ml aliquot of each fraction in TKMM buffer was dialyzed against 50 ml of the same buffer containing ^3H -cortisol in various concentrations for 48 hr at 0°C. Amount

of the bound hormone was then estimated by subtracting the outside counts from the inside ones in each 0.4 ml aliquots in duplicate. Protein was determined by the method of Lowry *et al.* (6) using bovine serum albumin as a standard protein.

Sucrose density gradient centrifugation

The cytosol labeled with ^3H -cortisol by the equilibrium dialysis was layered on the top of 4.9 ml of 5-20% linear sucrose density gradient prepared in TKMM buffer. Centrifugation was performed in a Beckman SW 50.1 rotor at 45,000 rpm for 16 hr. Bovine serum albumin was used as an internal marker.

Cortisol-binding activity of cytosol fraction obtained from granuloma in various stages

The 105,000 g supernatant fractions were prepared from 5, 10 and 22 day old granuloma. The binding assay for cortisol-binding protein was performed by the DEAE-cellulose filter method by Santi *et al.* (7) with slight modification.

Preparation of dissociated cells from carrageenin granuloma

Dissociated cells were prepared from five of 7 day old carrageenin granuloma by the method of Murota *et al.* (8). After slicing, the tissue was digested with a mixture of 0.05% collagenase and 0.05% trypsin in a phosphate buffered saline (PBS) at 37°C for 40 min, followed by adding 20% of fetal bovine serum in PBS to the medium to inactivate these enzymes. After pipetting, the dispersed cells were filtered through a 150 mesh platinum filter, and the dissociated cells were washed three times with serum free PBS. Contamination of serum transcortine to the cell fraction was thus successfully prevented. The dissociated cells were used for preparation of cortisol-binding protein.

RESULTS

Purification of cortisol-binding protein from the granuloma cytosol labeled with ^3H -cortisol in vitro

The 105,000 g supernatant fraction (Fraction I) obtained by ultracentrifugation from the granuloma cytoplasm was incubated with ^3H -cortisol at 0°C or 37°C and subjected to

FIG. 1. $(\text{NH}_4)_2\text{SO}_4$ fractionation to the Fraction I (the 105,000 g supernatant fraction of carrageenin granuloma cytosol). Thirty ml of Fraction I were previously incubated with 10 μCi of ^3H -cortisol at (a) 0°C or (b) 37°C for 1 hr.

stepwise fractionation with saturated $(\text{NH}_4)_2\text{SO}_4$. An aliquot of total radioactivity in the supernatant or the precipitate dissolved in TKMM buffer was counted for each fraction. As shown in Fig. 1, most of the labeled hormone appeared in the precipitate of 50–70% saturated $(\text{NH}_4)_2\text{SO}_4$ (Fraction II). It was then subjected to further purification by DEAE-cellulose chromatography after dialysis against TKMM buffer to eliminate excess $(\text{NH}_4)_2\text{SO}_4$, followed by labeling with ^3H -cortisol to saturate binding sites.

FIG. 2. DEAE-cellulose column chromatography of the Fraction II labeled *in vitro* with ^3H -cortisol. The Fraction II (19.5 ml) was incubated with ^3H -cortisol (0.25 $\mu\text{Ci/ml}$) applied on a DEAE-cellulose column (0.9 cm $\times$ 17.5 cm). The effluent was collected in 35 drops.

FIG. 3. DEAE-cellulose column rechromatography of the cortisol-binding protein fraction obtained from the first DEAE-cellulose column chromatography (Fig. 2). The fractions (tube no. 99, 100) from the first column effluence were pooled, dialyzed against TKMM buffer in order to remove NaCl , and labeled by equilibrium dialysis. Chromatography was done in the same way as mentioned in Fig. 2 except column size (0.9 cm $\times$ 11.5 cm).

The chromatogram of this fraction to DEAE-cellulose using a shallow gradient of NaCl (0.1–0.3 M) indicated two radioactive peaks (Fig. 2). Only the peak eluted near 0.12 M NaCl was revealed in the cortisol-binding protein fraction by equilibrium dialysis. This radioactive fraction was used as DEAE-cellulose-purified binding protein (Fraction III) for further experiments. Fraction III was subjected to dialysis against TKMM buffer and incubated with ^3H -cortisol once again, followed by rechromatography in the same way on the same column.

The absorbance and radioactive profiles on the salt gradient are shown in Fig. 3. The peak of radioactivity coincided with that of absorbance at 280 m μ and was confirmed to cortisol-binding protein.

Binding ability of cortisol to each fraction was examined. By DEAE-cellulose column chromatography the specific activity of the cortisol-binding protein was purified 32 fold to compare with the original 105,000 g supernatant fraction (Table 1).

TABLE 1. Purification of the binding protein for cortisol, as monitored by equilibrium dialysis

Fraction	Total protein (mg)	Specific binding activity (dpm bound/mg protein)	Fold purification
I	732	39,206	1
II	215	86,386	2
III	16	1,266,771	32

Fraction I: 105,000 g supernatant of the granuloma cytoplasm.

Fraction II: precipitate of 50–70% saturated $(\text{NH}_4)_2\text{SO}_4$ for the Fraction I.

Fraction III: DEAE-cellulose-purified binding protein.

TABLE 2. Effect of various unlabeled hormones on the binding ability of ^3H -cortisol to the binding protein

Unlabeled hormone added	Bound ^3H -cortisol	
	Count (dpm)	%
None	4,084	100
Cortisol	118	3
Androstenedione	669	17
Testosterone	759	18
17 β -Estradiol	2,484	61

Fraction III was diluted with TKMM buffer, incubated with ^3H -cortisol (0.25 $\mu\text{Ci}/\text{ml}$) for 1 hr at 0–4°C, and then dispensed to centrifugal tubes, 1 ml to each tube. Various unlabeled hormones at a concentration of 100 μg in 0.1 ml of ethanol were added. To the control tubes, 0.1 ml of ethanol was added. All the tubes were incubated for another 2 hr at 0°C. Bound cortisol was determined by the DEAE washing method (3).

Properties of the cortisol-binding protein in granuloma cytosol

The following experiments were performed using Fraction III. The optimum pH for binding was seen at pH 8.0 by the equilibrium dialysis method.

Effects of the other hormones were examined by the DEAE-washing method (3). The

high-salt washes of radioactivity adhering to the DEAE-cellulose were counted. Binding percentage is indicated in the last column of Table 2. The radioactivity of bound cortisol after the addition of unlabeled cortisol was completely lost, suggesting almost complete competition. Unlabeled testosterone was also more competitive than expected.

Cortisol-binding protein was treated with protease and RNase (Table 3). Both pronase E and trypsin inactivated the binding ability, while ribonuclease had little effect on the binding ability to labeled cortisol. This result suggests that the cortisol-binding ability was denatured by certain proteases.

Determination of dissociation constant for the cortisol-binding protein

The dissociation constant of the hormone-binding protein, "Kd" was estimated by the equilibrium dialysis method and calculated by Sherman *et al.*'s formula (9). As shown in Table 4, Kd was compared in the three fractions and estimated at about 1.0×10^{-9} M. The value is remarkably similar to the B-binder of cortisol receptor protein in rat liver cytosol (1.1×10^{-9} M⁻¹) reported by Beato *et al.* (4).

Sedimentation constant of cortisol-binding protein

Sedimentation constants of the Fraction I–III were determined by sucrose density gradient centrifugation. In addition, S values of the following 2 fractions were measured, DEAE-cellulose purified fraction of rat serum, reported to contain transcortine (11–15), and Fraction II obtained from dissociated cells of granuloma which were completely free from contamination by transcortine derived from serum and intercellular matrix. Each sample was labeled with ³H-cortisol by the equilibrium dialysis and S value was determined using BSA as internal standard marker.

As shown in Fig. 4 in all cases, those complexes sedimented close to BSA peak of the internal marker whose S value is generally regarded as 4.6 S. Therefore, S value of the complex in granuloma cytosol was estimated as 4–5 S. These results revealed that although the complex resembled transcortine it was not transcortine itself.

TABLE 3. Effect of various enzymes on the binding ability of ³H-cortisol to the binding protein

Enzyme	Bound ³ H-cortisol	
	Count (dpm)	%
None	1,524	100
Pronase E	134	9
Trypsin	139	9
Chymotrypsin	638	42
Ribonuclease	1,186	78

The experimental procedure was the same as in Table 2. In this case, instead of various unlabeled hormones, various proteases and RNase, 250 µg each, were incubated with the ³H-cortisol-binding protein complex at 5°C for 3 hr.

TABLE 4. Dissociation constant for each fraction

Fraction	K _d × 10 ⁹ (M)
I	1.04
II	0.88
III	1.00

Dissociation constant of each fraction was determined by the equilibrium dialysis method.

FIG. 4. Sucrose density gradient centrifugation of the ^3H -Fraction III and the others. These cortisol-binding proteins labeled by the equilibrium dialysis method were subjected to sucrose density gradient centrifugation as described in MATERIALS AND METHODS. (a) The Fraction III from carrageenin granuloma cytosol, (b) the Fraction II from dissociated cells (1.3×10^9 cells) of 7 day old carrageenin granuloma, (c) serum transcortine. Arrows indicate the absorbance peak at 280 $\text{m}\mu$ of bovine serum albumin as an internal marker.

DEAE-cellulose column chromatographic pattern of cortisol-binding protein from the granuloma cytosol in vivo labeled

Eight rats bearing granuloma were intravenously injected with ^3H -cortisol carried with 50 folds unlabeled carrier cortisol, the concentration of which was determined to be the most effective for *in vivo* pulse labeling the binding protein during 45 min (unpublished data). In this case, different from the *in vitro* labeling, the dialysis procedure was omitted considering the loss of *in vivo* labeled cortisol by exchanger or release. Fraction I was directly subjected to column chromatography. As shown in Fig. 5, the chromatogram was similar to that of *in vitro* labeling, the radioactive peak near the 0.12 M NaCl area was confirmed to be the cortisol-binding protein by the equilibrium dialysis.

^3H -cortisol binding activities of the cytosol fractions from granuloma in various stages

It was previously reported that the 5 day old granuloma was the most sensitive to

FIG. 5. DEAE-cellulose column chromatography of the cytosol fraction labeled with ^3H -cortisol *in vivo*. Twenty-five ml of Fraction I was directly applied on a DEAE-cellulose column ($0.9\text{ cm} \times 17\text{ cm}$). Other chromatographic conditions were as in Fig. 2.

TABLE 5. ^3H -Cortisol binding activity of cytosol fraction from the granulomas in various stages

Stage (days)	No. of animals	Pouch wall weight (g)	^3H -Cortisol bound (dpm/mg protein)	$K_d \times 10^9$ (M)
5	5	8.1	$27,110 \pm 1781$	0.8
10	5	7.8	$21,705 \pm 1301$	0.9
22	5	4.8	$9,498 \pm 347$	1.1

^3H -Cortisol binding activity was measured by the DEAE-cellulose filter assay method of Santi *et al.* (8). The values are shown as the mean $\pm$ S.E. Dissociation constant of the cytosol fraction on each stage was determined by the equilibrium dialysis method, and the differences were under statistically significant levels ($P > 0.05$).

gluco-corticoid, which could reduce the pre-existing granuloma. The sensitivity declined gradually with the age of the granuloma even though the dose of the drug was increased with age of the animal. (1, 10). Thus it was assumed that change in the cortisol binding ability may be related to the involution of granuloma by gluco-corticoid. Therefore, cortisol binding activities of the granuloma cytosol fractions at various stages of granuloma were examined to the specific binding radioactivity of ^3H -cortisol per mg protein. As shown in Table 5, the maximum binding ability was demonstrated on a 5 day old granuloma which is the so-called 'steroid sensitive stage'. Moreover it was clearly shown that the binding ability decreased with the increasing stage of granuloma. The dissociation constant by the method of equilibrium dialysis exhibited little or no change in all three stages.

DISCUSSION

When cortisol-binding protein from cytosol fraction of inflammatory tissue, the 8 day old granuloma, was prepared by fractionation of saturated ammonium sulfate and by

DEAE-cellulose column chromatography with a shallow salt gradient, the fractionation by ammonium sulfate for 105,000 g supernatant fraction gave the same result in two different conditions at 0°C and 37°C for 30 min (Fig. 1) despite the fact that the condition labeling hormone at 37°C for 30 min denatures the gluco-corticoid receptor (7). However, to prevent the binding protein from digesting with various proteases activated at 37°C, binding condition was fixed 0°C. By DEAE-cellulose column chromatography, free ^3H -cortisol and unnecessary protein were eliminated. As shown in Table 1, the binding specific activity was purified 32 fold in Fraction III in order to make a comparison with the original 105,000 g supernatant fraction. The cortisol-binding protein was assayed by the equilibrium dialysis method. Only 918 cpm were bound to the fraction eluted by initial buffer, while 434,507 cpm were bound to the fraction eluted by 0.12 M NaCl. So, the fraction eluted by 0.12 M NaCl was considered to be cortisol-binding protein fraction. The salt concentration required to elute the binding protein coincided with the result of cortisol B-binder from rat liver cytosol reported by Beato *et al.* (4).

On Fraction III, properties of the cortisol-binding protein from the inflammatory tissue were examined. First, optimum pH was indicated to be 8.0, which was similar to that of cortisol-binding protein from rat liver cytosol reported by Gopalakrishnan (3). Second, it was found that the cortisol-binding protein was denatured by various proteases, but not RNase (Table 3). It was also ascertained that DNase had no effect on the binding ability. In another experiment, the binding protein was indicated to be a specific binding protein for cortisol (Table 2). Since testosterone was also found to fairly compete with ^3H -cortisol, we examined the location of testosterone-binding protein by DEAE-cellulose column chromatography, and it was revealed to be eluted at lower salt concentration than 0.12 M at which concentration the cortisol-binding protein was eluted (Fig. 6). It was suggested

FIG. 6. DEAE-cellulose column chromatography of Fraction II labeled with ^{14}C -testosterone *in vitro*. Fraction II obtained from the granuloma cytosol was labeled with 1 μCi of ^{14}C -testosterone (spec. act. 59 mCi/mmmole) instead of ^3H -cortisol. The other conditions were as in Fig. 2.

that testosterone-binding protein could be eluted overlap with cortisol-binding protein.

The dissociation constant of cortisol-binding protein complex of granuloma cytosol was very similar to that of B-binder of cortisol receptor protein in liver cytosol ($1.1 \times 10^9 \text{ M}^{-1}$) reported by Beato *et al.* (4), but different from that of serum transcortine ($3.7 \times 10^{-11} \text{ M}$) which was estimated by the same method.

The sedimentation constant of cortisol-binding protein was very close to that of bovine serum albumin as the internal marker and rat serum transcortine (Fig. 4a-c). When intact granuloma tissue was used, contamination of serum transcortine was inevitable as the carrageenin granuloma tissue has many capillary vessels. To eliminate the contamination of serum transcortine, an attempt was made to disperse the granuloma tissue and collect dissociated cells only. The cortisol-binding protein was readily detected in the cytosol fraction of the dissociated cells. Furthermore, in our preliminary experiment by *in vivo* labeling, it was revealed that the same binding protein was detected even in the separated nucleoplasmic fraction which was completely free from the possibility of contamination of serum transcortine. From these findings, it may be concluded that there exists cortisol-binding protein specific to inflammatory tissue. A good correlation between the amount of the binding protein in the inflammatory tissue and anti-inflammatory effect of cortisol (Table 5) suggests that the cortisol-binding protein in the inflammatory tissue should play an important role in anti-inflammation mechanism.

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