Effect of receptor occupancy on [3H]dexamethasone binding to circulating leukocytes

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Abstract. To determine the effect of steroid occupancy of the glucocorticoid receptor on the measurement of total receptor concentration using Scatchard plot of [3H]dexamethasone (Dex) binding to circulating leukocytes, leukocytes were incubated with 10−7 M cortisol or 10−9 M Dex prior to [3H]Dex binding study. Total binding capacity calculated from Scatchard plot analysis of [3H]Dex binding was not significantly reduced after pre-incubation with cortisol. However, total binding capacity was significantly lower after pre-incubation with Dex. These data suggest that total glucocorticoid receptor concentration can be measured from a 3 h incubation of leukocytes obtained from untreated patients.

Certain disorders that suggest a lack of response to steroid hormones may be explained by abnormality in steroid receptor number (Gehring et al. 1971; Amrhein et al. 1976). There are few data relevant to disorders of glucocorticoid receptor in man due, in part, to the difficulty of obtaining suitable samples from patients. Peripheral blood is an ideal material for the clinical studies requiring serial analysis of the glucocorticoid receptor. We have characterized the glucocorticoid receptor in the circulating leukocytes (Murakami et al. 1979a,b) and have shown that glucocorticoid receptor can be quantified accurately. However, the glucocorticoid receptors in the circulating leukocytes are presumptively occupied by endogenous cortisol. The present experiments were designed to analyze the effect of endogenous cortisol on the measurement of glucocorticoid receptors using [3H]dexamethasone (Dex).

Materials and Methods

Reagents

[6,7-3H]Dexamethasone (48.0 Ci/mmole) was purchased from New England Nuclear. Unlabelled steroids were purchased from Sigma. RPMI-1640 containing 100 U/ml of penicillin and 100 μg/ml of streptomycin was obtained from GIBCO. Human serum albumin (Miles Laboratory, Inc.) was added to RPMI-1640 (1% final concentration) prior to use. Phosphate buffered saline (PBS) consisted of 0.15 M NaCl and 6.7 mM phosphate (pH 7.2), and was used at 0–4°C. Lysis buffer consisted of 0.155 M NaCl, 10 mM K2HPO4 and NaH2PO4, and 0.1 mM EDTA (pH 7.2). Ten ml of Aquasol (New England Nuclear) was added to vials and radioactivity (tritium) was measured in a Packard Tri-Carb liquid scintillation spectrophotometer at 40% efficiency.

Pre-incubation of buffy coat with steroids

All procedures were performed at 0–4°C unless otherwise noted.
Heparinized peripheral blood from a normal female goat (1–2 years of age) was diluted 1:3 with PBS, and centrifuged at 2000 × g for 3 min. Theuffy coat was removed and suspended in PBS. Cell suspension was centrifuged at 250 × g for 10 min, and the supernatant was discarded. This washing procedure was repeated twice. The pellet was suspended in 100 ml of RPMI-1640 and divided into 2 culture flasks (75 cm²). Five µl of ethanol solution of 10⁻⁵ M Dex or 10⁻³ M cortisol was added to one of these flasks, and 5 µl of ethanol was added to the other flask as a control. The cell suspensions were incubated at 20°C for 2 h. After the incubation, the cell suspensions were diluted 1:5 with lysing buffer and centrifuged at 450 × g for 10 min, and the leukocyte pellet was suspended in PBS and centrifuged at 250 × g for 10 min. This washing procedure was repeated twice, and the cells were suspended in RPMI-1640.

Preparation of total leukocytes

Buff-coated prepared from heparinized peripheral blood was diluted 1:3 with PBS, and 1 volume of this suspension was mixed with 4 volumes of lysing buffer. After the erythrocytes were lysed, the cell suspension was centrifuged at 450 × g for 10 min, and the leukocyte pellet was suspended in PBS and centrifuged at 250 × g for 10 min. This washing procedure was repeated twice, and the cells were suspended in RPMI-1640.

Intact cell binding study

Cell counts were adjusted to 1 × 10⁷/ml and 1.0 ml of this suspension was added to all tubes which had previously received 1.0 ml of RPMI-1640 containing various concentrations of [³H] Dex ranging from 0.1 to 40 nM with or without a 100-fold molar excess of unlabelled Dex. All tubes were incubated under 5% CO₂ and 95% air at 20°C in a Dubnoff shaking incubator. After 15 h, viability was maintained, and cell counts were not significantly reduced. After incubation, 5 ml of PBS was added to all tubes, and the tubes were centrifuged at 250 × g for 10 min. The supernatant was discarded, the cells were re-suspended in 5 ml of PBS and centrifuged as above, and this washing procedure was repeated. Following the final wash, 1.0 ml of PBS was added to each tube, and the pellets were thoroughly suspended, transferred to counting vials, and the radioactivity was measured.

Experiments and Results

Non-specific binding to intact cells

Binding studies were performed for 3 h at 20°C as described above. Non-specific binding was determined using a 100-fold molar excess of non-labelled Dex.

[³H]Dex bound to intact leukocytes was plotted as non-specific binding vs. concentration of [³H]Dex in Fig. 1. Non-specific binding increased linearly between 0 to 20 nM of [³H]Dex. However, at concentrations higher than 20 nM of [³H]Dex (2 μM non-labelled Dex), the increase in non-specific binding became non-linear.

Effect of pre-incubation of leukocytes with glucocorticoids

Binding studies for [³H]Dex using intact cells pre-incubated with unlabelled Dex or cortisol were performed for 3 h as above. Specific binding to saturable receptor was calculated as the difference between total and non-specific binding. Data were analysed using a computer program for routine analysis of Scatchard plots (Murakami et al. 1979a).

The Scatchard plots were consistent with a single high affinity binding site independent of pre-incubation with steroids as shown in Figs. 2 and 3. The dissociation constants (Kₐ) and total binding capacities are shown in Table 1. The Kₐ's for the groups pre-incubated without steroid was similar to the
Effect of pre-incubation of leukocytes with $10^{-7}$M cortisol. Buffy coat was incubated with or without $10^{-7}$M cortisol for 2 h at 20°C. After the incubation, red cells were lysed. leukocytes were washed with PBS and approximately $10^7$ leukocytes were incubated with various concentrations of $[3^H]$Dex with or without unlabelled Dex. After 3 h incubation at 20°C, cells were washed with PBS and $[3^H]$Dex bound to cells was measured. Specific binding to leukocytes pre-incubated with or without cortisol was plotted vs. concentration of $[3^H]$Dex. Specific binding was replotted using Scatchard plots (inset).

Effect of pre-incubation of leukocytes with $10^{-9}$M Dex. Buffy coat was incubated with or without $10^{-9}$M Dex at 20°C, and $[3^H]$Dex bound to leukocytes was measured as described in the legend to Fig. 2. Specific binding to leukocytes pre-incubated with or without Dex was plotted vs. concentration of $[3^H]$Dex. Specific binding was replotted using Scatchard plots (inset).
Table 1. Effect of pre-incubation with or without steroids.

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Kd (nM)</th>
<th>Binding capacity (fmole/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-7 M cortisol</td>
<td>6.7 ± 1.5 (SD)</td>
<td>7.0 ± 1.1 (SD)</td>
</tr>
<tr>
<td>without steroid</td>
<td>3.4 ± 0.8</td>
<td>7.4 ± 1.0</td>
</tr>
<tr>
<td>10^-4 M dexamethasone</td>
<td>4.8 ± 0.1</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>without steroid</td>
<td>3.8 ± 0.1</td>
<td>7.2 ± 0.5</td>
</tr>
</tbody>
</table>

Data are based on the experiments of Figs. 2 and 3.

Kd's reported previously (Murakami et al. 1979a,b). Total binding capacity for the cells pre-incubated with cortisol was not significantly different from the value for the cells pre-incubated without steroid. However, binding capacity for the cells pre-incubated with Dex was significantly lower than the value for the cells pre-incubated in the absence of steroid.

Change of Scatchard plots with increased incubation times.

Leukocytes prepared from theuffy coat were divided into four groups, and [3H]Dex binding studies were performed as above. These groups were incubated for 2, 4, 8, and 15 h at 20°C. Scatchard analysis revealed a single component specific binding for each group as shown in Fig. 4. The apparent Kd decreased with longer incubation times. The binding capacity, however, was constant at all time periods.

Discussion

The total glucocorticoid receptor concentration can be quantified accurately using intact leukocytes from peripheral blood. Pre-incubation of cells with 10^-7 M cortisol did not significantly change the apparent receptor concentrations. This suggests that total receptor concentration calculated from Scatchard plots of [3H]Dex binding to leukocytes would not be affected by the endogenous steroid occupancy of receptors.

As we have previously described (Murakami et al. 1979a), analysis of the kinetics of steroid dissociation from mononuclear leukocytes revealed 2 components at 20°C. We suggested that the slowly dissociating component was the 'activated' steroid receptor complex generated by the temperature dependent process. Accordingly, [3H]Dex interacts with glucocorticoid receptor of at least 3 different forms at 20°C after the pre-incubation of leukocytes with cortisol or Dex: these are free non-activated receptors, non-activated occupied receptors, and activated occupied receptors. When receptors are present in 2 or more isomerizing forms, there is a linear Scatchard plot (Klett et al. 1973; Ross et al. 1977). This isomerizing model is the likely explanation for a linear Scatchard plot in spite of the co-existence of at least 3 forms of receptors at 20°C after pre-incubation with cortisol or Dex. The apparently higher Kd (lower affinity) of the leukocytes pre-incubated with glucocorticoids is presumably due to the occupancy of the receptors by the steroids used for pre-incubation. Total receptor concentration calculated for the leukocytes pre-incubated with 10^-9 M Dex was significantly lower than that for the leukocytes incubated without steroid. The half time of the
dissociation of [3H]Dex-receptor complex was about 12 h after activation at 20°C (Murakami et al. 1979a). Thus a 3 h incubation with [3H]Dex would be too short to establish exchange, and the total receptor concentration measurable would be low.

In this study, non-specific binding was calculated as [3H]Dex bound in the presence of a 100-fold molar excess of unlabelled Dex. This analysis permits the estimation of the number of high affinity receptor sites i.e. specific binding, by subtracting non-specific binding from total binding. It has been assumed that non-specific binding increases linearly as the concentration of [3H]Dex increases. However, non-specific binding was not linear at concentrations higher than 20 nM of [3H]Dex in the presence of a 100-fold molar excess of unlabelled Dex. Thus the linear range of the non-specific binding of [3H]Dex is limited, probably due to saturation of the non-specific sites by the very high Dex concentrations employed.

The values of the total binding capacity were constant in the presence of the shift of Ka with increasing time of incubation at 20°C, and Scatchard plot analysis of each time of incubation showed a single component of binding sites. These results indicate that even if the reaction between [3H]Dex and receptor has not reached equilibrium, receptor concentration can be calculated using Scatchard plot analysis. Furthermore, the effect of endogenous steroid did not significantly alter the value for the receptor concentration calculated from Scatchard plot of a 3 h incubation of leukocytes at 20°C. Thus glucocorticoid receptor concentration can be calculated from a Scatchard plot of [3H]Dex binding to intact leukocytes from patients with normal amounts of circulating cortisol.

References


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