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Characterization of Glucocorticoid Binding Capacity in Human Mononuclear Lymphocytes: Increase by Metyrapone is Prevented by Dexamethasone Pretreatment

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Abstract

Autoregulation of receptor systems by their own ligands is a well established biological phenomenon. While down-regulation of the glucocorticoid binding capacity by glucocorticoids has been shown in animals and humans, data on up-regulation processes in humans are lacking. To further explore glucocorticoid receptor plasticity in relation to endogenous ligands, glucocorticoid binding parameters were assessed in 15 healthy controls before and after oral administration of 1.5 g metyrapone with and without dexamethasone pretreatment. Administration of metyrapone resulted in blockade of the feedback of the hypothalamic-pituitary-adrenal system as shown by the rise in adrenocorticotropin levels, while pretreatment with 1 mg dexamethasone completely suppressed adrenocorticotropin concentrations. Glucocorticoid binding sites per lymphocyte exhibited an increase of 63% following metyrapone administration, which was prevented by dexamethasone pretreatment. Comparison of morning and afternoon glucocorticoid binding sites per cell in 11 healthy volunteers further revealed a diurnal rhythm of glucocorticoid receptor sites. These data suggest that human lymphocyte glucocorticoid receptors are under autoregulatory control.

Glucocorticoid receptors (GR) have been identified in numerous mammalian and human tissues including brain (1–3), liver (4), lung (5), skin (6, 7), and peripheral blood cells (8–10). Although several lines of evidence suggest an immunosuppressive effect of glucocorticoids with therapeutic implications (11), the exact mechanism of glucocorticoid action in relation to immune function remains unsolved. Glucocorticoids have been shown both to inhibit lymphocyte proliferation (11) and to induce redistribution of circulating leukocytes to the bone marrow (11).

Exposure to higher doses of glucocorticoids can down-regulate the GR in animals (12) and humans (13, 14). In addition, investigations on GR pharmacology in human leukocytes at different times of day (10, 15, 16) or in adrenocortical disorders (10, 17, 18) have provided conflicting evidence for a diurnal rhythm of GR (15) and decreased GR numbers in adrenal insufficiency (18) or Cushing's syndrome (19), which others were unable to confirm (10, 17).

Down-regulation of GR by glucocorticoids has been relatively easy to study in either experimental animals or man, while data on up-regulation phenomena are available only from animals after adrenalectomy (20). The present study was designed to explore whether an increase in GR binding capacity can be achieved by metyrapone (MET) administration in healthy volunteers mimicking the effect of adrenalectomy and whether this can be reversed by exogenous glucocorticoids.

Results

Pharmacological characteristics of the GR

Linear regression analysis was performed on all saturation experiments; a representative Scatchard plot is shown in Fig. 1. Saturation experiments clearly revealed only one binding site. The Hill coefficient was close to unity (1.00 ± 0.001), indicating an absence of cooperativity of the binding site.

Specific binding of 6 nM [^{3}H]dexamethasone ([^{3}H]DEX) was completely displaced by the pure glucocorticoid agonist RU 28362 with a K_i value of 7.8 ± 2.1 nM, by DEX with a K_i value of 13.4 ± 1.7 nM, and by cortisol with a K_i value of 117 ± 31 nM, respectively, under equilibrium conditions in three different experiments.

There was a linear relationship between absolute GR and lymphocyte number in the assay.

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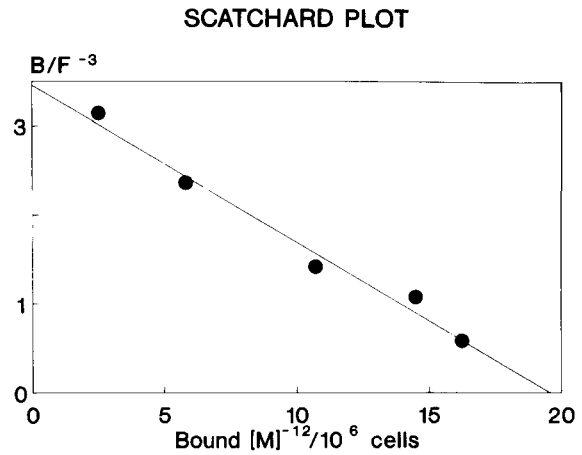

FIG. 1. Scatchard plot of a representative saturation experiment of specific [^{3}H]dexamethasone binding to human mononuclear leukocytes. Specifically bound/free [^{3}H]dexamethasone is plotted versus specifically bound [^{3}H]dexamethasone.

Glucocorticoid binding capacity in relation to endogenous glucocorticoid levels in vivo

Assessment of diurnal variation of GR binding pharmacology revealed a marked increase of GR sites per cell in the afternoon (2542 ± 504 versus 4060 ± 1184 receptor sites per cell; $t = 4.33$, $df = 10$, $P < 0.001$) contemporaneous with a pronounced fall in plasma cortisol (557.3 ± 141.5 versus 273.1 ± 144.5 nmol/L; $t = 5.55$, $df = 10$, $P < 0.001$), while the K_d did not change significantly (8.6 ± 2.7 versus 10.3 ± 3.3 nM).

Administration of MET resulted in blockade of 11ß-hydroxylase activity shown by the rise in adrenocorticotropin (ACTH) (Fig. 2; $t = 4.5$, $df = 14$, $P < 0.001$), which was prevented by DEX pretreatment. MET administration also increased the GR sites per cell by 63% within 7 h (Fig. 3; $t = 3.98$, $df = 14$, $P < 0.001$), and this effect was blocked by low dose DEX pretreatment. Depletion of endogenous glucocorticoids by MET and subsequent replacement by DEX did not significantly alter the K_d (Fig. 4).

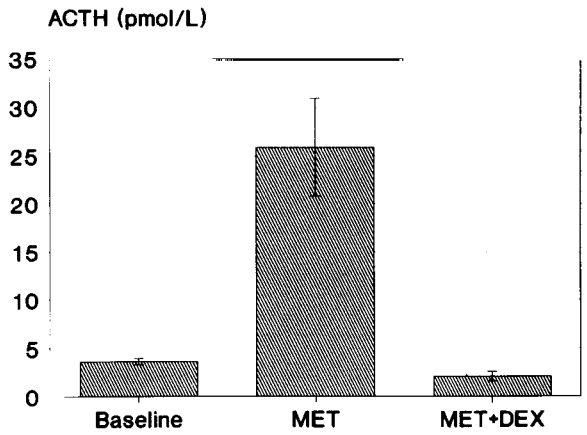

FIG. 2. Mean ± SE plasma adrenocorticotropin (ACTH) concentrations at 1600 h on three consecutive days in 15 healthy controls under baseline, metyrapone (MET) and metyrapone plus dexamethasone (MET + DEX) pretreated conditions. ACTH levels are significantly increased by MET administration ($t = 4.5$, $df = 14$, $P < 0.001$). This increase is prevented by DEX pretreatment.

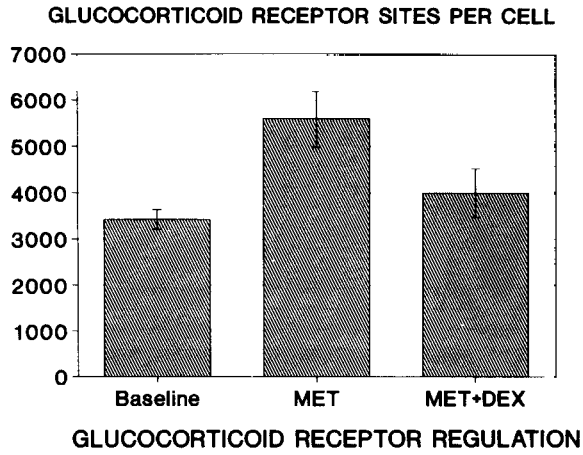

FIG. 3. Mean ± SE plasma glucocorticoid receptor (GR) binding sites per cell at 1600 h on three consecutive days in 15 healthy controls under baseline, metyrapone (MET) and metyrapone plus dexamethasone (MET + DEX) pretreated conditions. GR sites per cell are significantly increased by MET administration ($t = 3.98$, $df = 14$, $P < 0.001$). This increase is prevented by DEX pretreatment.

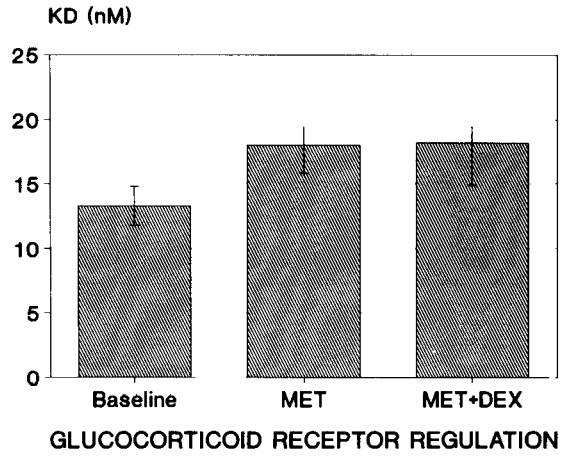

FIG. 4. Mean ± SE K_d values at 1600 h on three consecutive days in 15 healthy controls under baseline, metyrapone (MET) and metyrapone plus dexamethasone (MET + DEX) pretreated conditions.

No relationship was found between age, sex, body weight, ACTH, or baseline cortisol levels (223.7 ± 75.6 nmol/L) and GR binding parameters under the conditions studied.

Discussion

Although previous investigations of a possible diurnal rhythm of GR binding parameters have yielded contradictory results (10, 15, 16), the present study demonstrated clear diurnal rhythm of GR sites with no apparent change in GR affinity. Interestingly, we did not observe a significant correlation between GR sites and ACTH or cortisol levels, suggesting that plasma concentrations of endogenous glucocorticoids at the time of the GR assay do not control the GR sites. Estimation of cortisol profiles several

hours prior to that of GR sites however might clarify the relationship of GR sites and endogenous glucocorticoids.

We demonstrated an increase in GR activity binding 7 h following MET pretreatment. At this time ACTH levels had also increased dramatically due to the lack of feedback action of 11ß-hydroxylated endogenous glucocorticoids. This increase could be prevented by prior administration of the synthetic glucocorticoid DEX. Since glucocorticoids probably need several hours to exert a notable influence on GR regulation, this might explain the lack of correlations between GR sites and plasma cortisol concentrations at the time of the GR assay.

Our results in humans accord with those of animal studies and recent *in vitro* investigations at the protein and DNA level. A rise in GR sites in the rat brain has been observed following adrenalectomy (20), while exposure to glucocorticoids results in a down-regulation of GR sites of various cell lines (13, 14, 21). While glucocorticoids reduce GR mRNA (22, 23) and GR protein (24) within several hours following binding of GR to GR cDNA, receptor degradation may also be involved as an autoregulatory post-translational control mechanism independent of GR protein synthesis (21, 25).

Although changes in GR binding do not necessarily reflect changes in receptor mass, since the results of binding studies are dependent on the availability of receptors for exchange with radiolabelled tracer, an exact estimation of GR sites after sufficient dissociation of endogenous glucocorticoids has been claimed (13). The use of monoclonal antibodies and mRNA probes (24) may help to discriminate between transcriptional and post-translational phenomena and further our understanding of human physiology.

Materials and Methods

Subjects

Fifteen subjects (three men and twelve women) participated in the study on a voluntary basis. Their mean age and body weight were 47.3 ± 6.9 ($\pm$SD) years and 68.8 ± 9.9 kg, respectively. Exclusion criteria included illness, intake of medication, alcohol or nicotine abuse and stressful life events prior to the investigation. Five women were tested during the midluteal phase of the cycle and seven were postmenopausal. Diurnal variation of GR characteristics was assessed in an additional 11 healthy volunteers (six men and five women) with a mean age of 35.2 ± 15.2 years and a mean body weight of 67.2 ± 8.9 kg.

Test protocol

All subjects were admitted to a sleep laboratory at least 1 h prior to drug administration or blood sampling. The test protocol was approved by the ethical committee of the University of Würzburg. For determination of GR binding characteristics and hormone data 50 ml blood samples were collected at 1600 h into prechilled plastic tubes containing EDTA on three consecutive days. On Day 1 baseline values were obtained. On Day 2 the subjects were pretreated with 1.5 g MET at 0900 h administered orally with milk to avoid severe gastric symptoms. One mg DEX was given orally at 2300 h. On Day 3 administration of 1.5 g MET was performed as described for the day before. Twelve of the 15 subjects experienced a transient dizziness and a hot flush, which disappeared within 45 to 60 min.

For the evaluation of diurnal GR binding parameters blood samples were obtained twice at 0800 h and 1600 h.

Chemicals

[^{3}H]DEX ([1,2,4,6,7-^{3}H]dexamethasone, specific activity 84 Ci/mmol) was obtained from Amersham, UK. Unlabelled DEX, cortisol, phosphate-buffered saline (PBS) and sodium metrizoate-Ficoll were obtained from Sigma (St. Louis, MO, USA), and RU 28362 from Roussel-Uclaf (Paris, France). Rotiszint 22 was obtained from Roth (Karlsruhe, West Germany). The incubation buffer consisted of PBS with 5 mM D-glucose added. Stock solutions of unlabelled steroids (50 μM) were prepared in incubation buffer containing 5% ethanol.

Preparation of cells

A mononuclear cell fraction was prepared by sodium metrizoate-Ficoll density gradient centrifugation (26). Cells were washed two times in PBS for 10 min, incubated for 60 min at 37 °C, and then washed again to allow dissociation of endogenous hormone. Endogenous cortisol levels after the washing procedure were below the detection limit of the assay. The final concentration of cells was determined using a Coulter Counter (Model S5; Coulter Electronics Ltd, England). Viability of cells exceeded 95%, as judged from their ability to exclude trypan blue. Contamination by erythrocytes was less than 10%, contamination by granulocytes and monocytes was less than 8% and did not differ between the three test days.

Binding assay

Binding experiments were carried out at 37 °C in plastic microtitre plates in a total volume of 0.25 mL. The displacing compound (10 μM unlabelled DEX) was added immediately prior to the addition of [^{3}H]DEX to determine non-specific binding. In saturation studies increasing concentrations of [^{3}H]DEX from 1 to 40 nM were used. Saturation experiments were performed at equilibrium after 90 min incubation. After incubation bound ligand was separated from free ligand by rapid filtration through Scatron filters with a Titertek cell harvester by two 5-s washes with assay buffer at room temperature. The filters were transferred into plastic vials, 5 mL of a toluene-based scintillation cocktail was added (Rotiszint 22) and they were monitored for tritium in a Beckman LS 1801 counter at about 54% efficiency. All samples were assayed in triplicate with a variation within a single experiment of less than 7%.

Hormone assays

ACTH was measured by a newly developed immunoradiometric assay supplied by the Nichols Institute (San Juan Capistrano, CA, USA), which does not require extraction procedures (27). A soluble sandwich complex is formed by a [^{125}I]labelled monoclonal antibody directed against N-terminal ACTH and a biotin-coupled polyclonal antibody against C-terminal ACTH. The sandwich complexes are bound by adding avidin-coated plastic beads. Unbound components are washed away and the radioactivity bound to the solid phase is monitored in a gamma-counter. The lower detection limit was 1.5 pmol/L, and the intra- and interassay coefficients of variation were 3% and 6.8%, respectively.

Cortisol was measured by a direct RIA (28). The lower detection limit was 25 nmol/L, and the intra- and interassay coefficients of variation were 5% and 9%, respectively. As there is cross-reactivity of most cortisol antibodies with 11-deoxycortisol (about 15% in our assay), assessments of cortisol values following MET application would not be accurate and were therefore excluded from further analysis.

Data analysis

Preliminary estimates of binding parameters from saturation experiments were provided by the EBDA program (29). Final estimates of binding parameters were determined with a computerized non-linear, least-square regression analysis (30). This weighted curve fitting program assumes binding according to the law of mass action to independent classes of binding sites.

The results are expressed as the mean $\pm$SD, and as the mean $\pm$SE in Figs. 2 to 4.

Data were analysed using the *t*-test for paired samples and Pearson's product moment correlation. All significance levels are two-tailed.

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