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REGULATION OF GLUCOCORTICOID RECEPTORS IN HUMAN MONONUCLEAR CELLS: EFFECTS OF GLUCOCORTICOID TREATMENT, CUSHING'S DISEASE AND KETOCONAZOLE*

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Summary—Glucocorticoid receptors (GcR) were determined by a whole cell assay in human mononuclear leukocytes (hMNL) from control subjects, patients receiving glucocorticoid therapy for systemic diseases and Cushing's disease patients with or without ketoconazole therapy. Prolonged corticosteroid treatment resulted in down-regulation of GcR, while the mean level of GcR in Cushing's disease was normal. In this group, however, receptor levels and morning plasma cortisol values showed a negative correlation, indicating a subtle down-regulatory effect. Furthermore, GcR were unaltered after these patients received ketoconazole, in spite of a marked reduction in morning plasma cortisol and urinary free cortisol. We also observed that ketoconazole was a weak competitor of GcR in intact cells, although it significantly inhibited [^{3}H]dexamethasone binding in cytosolic preparations from rat tissues. The results suggested that GcR in hMNL are down-regulated by synthetic steroids given *in vivo*, but they showed very mild down-regulation in hypercortisolemic patients suffering from Cushing's disease. Finally, we did not observed either up-regulation or antagonism of GcR by ketoconazole treatment, at the time that cortisol levels of patients with Cushing's disease were reduced. This indicates that the beneficial effects of ketoconazole in Cushing's disease are due to adrenal cortisol suppression and not to interaction with GcR of target cells, and that the process of GcR regulation in hMNL is a complex phenomenon awaiting further elucidation.

INTRODUCTION

It has been demonstrated that the glucocorticoid receptor (GcR) is regulated by its ligand in cell lines in culture as well as in living animals [1–4]. In normal human subjects, administration of both synthetic and natural adrenal hormones resulted in a 30% decrease in binding capacity of peripheral lymphocytes without changes in binding affinity towards [^{3}H]dexamethasone (DEX) [5]. Suggestions for down-regulation of GcR have also been provided by additional studies in normal volunteers receiving high-dose DEX during 24 h [6] and by the decreased receptor concentration observed in anorexia nervosa, a disease which is usually accompanied by hypercortisolism [7].

However, data regarding GcR in human mononuclear leukocytes (hMNL) of patients with adrenal dysfunction have yielded conflicting results. Thus, in hMNL from subjects with cortisol hypersecretion due to Cushing's disease [8, 9] and in the hypercortisolism of depressed patients [10], GcR appeared in the normal range. In adrenal insufficiency, on the other hand, some authors [8] have not found changes in GcR concentration, while others [11] observed a decrease in binding; in the latter case, GcR did not up-regulate in spite of a marked reduction in adrenal secretory products.

Considering these contradictory data, we have performed a series of studies involving human subjects with the purpose of establishing the response of GcR of hMNL to variations in circulating glucocorticoids. We have considered two opposing conditions, such as: (a) patients with high glucocorticoid levels as a consequence of endogenous hypersecretion as in Cushing's disease, or after exogenous corticosteroid

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administration for systemic diseases and (b) patients whose high cortisol levels were reduced by the administration of ketoconazole. This drug is an imidazole derivative used as an antimicotic agent and it has a well-known additional inhibitory effect on adrenal steroid biosynthesis by blocking the cytochrome *P*-450 enzymes [12]; by this virtue, it has been successfully used in the treatment of adrenal hyperfunction [13, 14]. Furthermore, and in view of the reported antagonistic activity of this agent at the GcR level in rat tissue hepatoma cell cytosol [15], we studied whether this action could partially account for the clinical improvement observed in Cushing's disease patients.

EXPERIMENTAL

Subjects

The control group was composed of 4 women and 6 men aged between 18–48 years, without a clinical history of endocrine diseases and free of medication. The group of patients receiving prolonged corticosteroid therapy was composed of 9 subjects with systemic diseases such as: viral pneumonitis [1], bronchial asthma [2], psoriasis [1] and vulgar pemphigus [5]. They received 20–80 mg/day of prednisone orally for 2 months–3 years. Nine Cushing's disease patients were available for study, 8 women and 1 man, aged between 17–46 years. In all these patients, the disease was confirmed by routine laboratory methodology, including lack of circadian rhythm of cortisol, elevated basal plasma and urinary-free cortisol levels, lack of inhibition with low-dose DEX tests and at least a 50% suppression of cortisol values with 8 mg DEX. Abdominal CAT scan and/or adrenal scintigram with 19-[^{131}I]-cholesterol showed bilateral hyperplastic adrenal glands. Radiological image of the sella turcica was performed, with CAT scanning being pathological in 2 out of 9 cases. Four additional patients with Cushing's diseases were studied before and after ketoconazole therapy in the doses of 400–600 mg/daily during 1 month, in 2 divided doses.

Glucocorticoid receptor assay in hMNL

The method of Kontula *et al.* [8] was adapted to our laboratory [9]. 50 ml Of heparinized blood were withdrawn at 8 a.m. and subjected to centrifugation on a Ficoll–Hypaque gradient. After subsequent washing procedures were PBS, pH 7.4, a cellular suspension containing $5–10 \times 10^6$ cell/ml, showing 95% viability by trypan blue-exclusion test, was preincubated in MEM containing 25 mM Hepes buffer, pH 7.4, for 30 min at 37°C. This step was taken in order to dissociate any endogenous ligand bound to GcR. Additionally, and to avoid further GcR occupancy by exogenous hormone, determination of GcR was performed after 24 h of the last intake of corticosteroid therapy. The cell suspension (in duplicate) was resuspended in MEM, pH 7.4, containing 0.6 mg/ml glutamine and aliquots were incubated with 50 nM [^{3}H]DEX in the presence or absence of 100-fold nonradioactive DEX for 30 min at 37°C, with gentle shaking. After three washings with PBS and centrifugation, the final pellet was resuspended in 0.2 ml PBS and added to 10 ml scintillation fluid composed of Triton-X100 in toluene (30:70, v/v). Samples were counted in a Packard-Tricarb spectrometer at an efficiency of 33%. Specific binding was obtained by the difference between total and nonspecific binding. GcR concentration was expressed as number of sites per cell (No. sites/cell).

For *in vitro* determination of ketoconazole antagonistic activity, hMNL cells prepared from healthy blood donors were incubated with 50 nM [^{3}H]DEX in the presence or absence of 20 or 100 μM ketoconazole. The competitor was dissolved in ethanol and evaporated with subsequent redilution to 10 μl ethanol, adding the same volume of vehicle to the control incubations.

Glucocorticoid receptors in cytosolic fractions

A previously published procedure was used in these experiments [16]. Male Sprague–Dawley rats (250–300 g) were adrenalectomized 2–3 days before sacrifice. After decapitation, the liver, thymus and hippocampus were excised and homogenized in 2–3 vol of TEMGMo buffer (10 mM Tris–HCl, 1.5 mM EDTA, 2 mM mercaptoethanol, 10% glycerol, 20 mM sodium molybdate, pH 7.4) and centrifuged at 105,000 *g* for 60 min. Aliquots of the resulting cytosol were incubated with 20 nM [^{3}H]DEX for 20 h at 0–4°C. Receptor-bound and free hormones were separated on Sephadex LH-20 minicolumns. The column eluates containing bound hormone were collected into vials and radioactivity was determined by liquid scintillation spectrometry. Ketoconazole was added as mentioned above for hMNL cells, and results were expressed as % inhibition of [^{3}H]DEX binding.

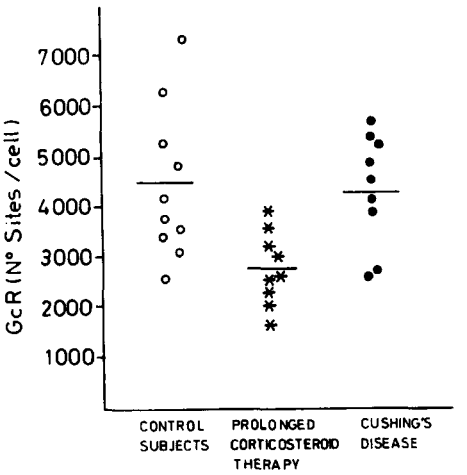

Fig. 1. Determination of GcR in hMNL from 10 control subjects, 9 patients receiving prolonged corticosteroid therapy (20–80 mg/day of oral prednisone during 2 months–3 years) and 9 patients with Cushing's disease. Each point represents an individual case, and the mean of the group is indicated by the horizontal line. GcR were similar in the control and Cushing's groups, whereas a significant reduction was obtained in the glucocorticoid-treated group ($P < 0.02$).

Materials

[^{3}H]DEX (sp. act. 49.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Ketoconazole was a gift from Janssen Laboratories (Argentina). Plasma cortisol was determined by a RIA kit from DPC (U.S.A.).

RESULTS

Glucocorticoid receptors in peripheral cells

In control subjects, levels of GcR in hMNL cells measured 4473 ± 476 sites/cell (Fig. 1). there was no difference between males (4250 ± 539, $n = 6$) and females (4806 ± 957, $n = 4$); therefore, data for both sexes were pooled for statistical evaluations. In three instances in which saturation analysis was carried out, K_d averaged 2 nM. As also shown in Fig. 1, patients receiving exogenous glucocorticoids for systemic diseases, presented a significant reduction of GcR to 3475 ± 453 sites/cell ($n = 9$, $P < 0.02$ vs the control group), with K_d remaining in the control range: 1.5 mM. In contrast, hypercortisolemic patients with Cushing's disease showed normal levels of GcR: 4425 ± 364 site/cell ($n = 9$) and normal K_d (2.5 nM).

Thus, the apparent down-regulation of GcR observed in patients receiving corticosteroids was not found in subjects with endogenous hypercortisolemia. However, when data for GcR levels in the Cushing's disease group was contrasted with the respective morning levels of plasma cortisol, a significant negative correlation emerged (Fig. 2), while no correlation was found for control subjects. These results suggest that a subtle down-regulation of GcR was present only in patients containing the highest levels of plasma cortisol.

Effects of ketoconazole on glucocorticoid receptor levels

In these studies, four patients with stigmata and biochemical characteristics of Cushing's disease received ketoconazole treatment, as stated in the Experimental section. Determination of plasma and urinary-free cortisol before and 1 month following continuous therapy with this drug demonstrated its effectiveness as

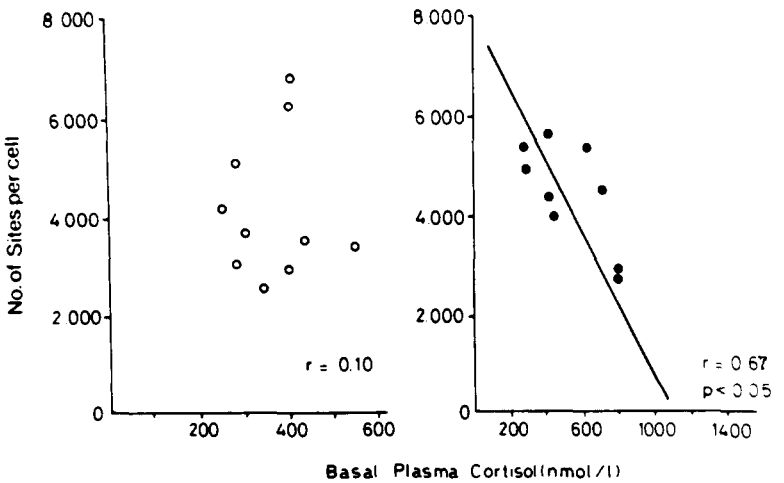

Fig. 2. Correlation between levels of plasma cortisol in the morning and GcR in hMNL. No correlation between these two variables was obtained in control subjects (left-hand panel), whereas a statistically significant negative correlation was found in patients with Cushing's disease (right-hand panel, $r = -0.67$, $P < 0.05$). Reproduced from Pardes *et al.* [9].

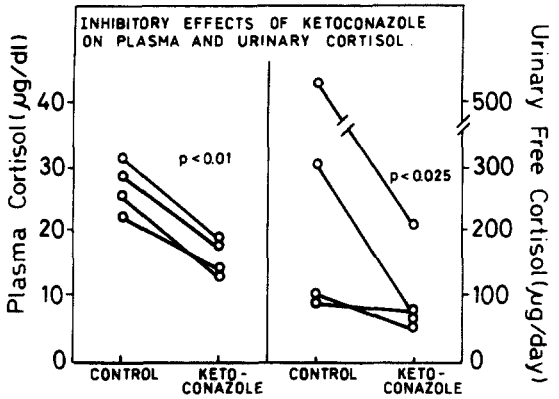

Fig. 3. Effectiveness of ketoconazole treatment to steroid levels in 4 patients with Cushing's disease. Plasma cortisol (μg/dl, left-hand panel) and urinary-free cortisol (μg/day, right-hand panel) were measured before (control) or after administration of 400–600 mg/day of ketoconazole during 1 month. A significant reduction in steroid levels was detected in all four cases ($P < 0.01$ for plasma corticol, $P < 0.025$ for urinary cortisol).

an inhibitor adrenal steroid secretion. Thus, plasma cortisol levels were reduced from 27.05 ± 1.82 to 16.02 ± 1.14 μg/dl ($P < 0.01$), with a reduction urinary-free cortisol from 259.0 ± 117 to 103.6 ± 41 μg/day ($P < 0.025$), as shown in Fig. 3. In the same patients, GcR levels in hMNL cells remained at 3996 ± 378 sites/cell following ketoconazole, from a control value to 4428 ± 527 (Fig. 4).

The unchanged GcR levels in hMNL cells after ketoconazole therapy suggested lack of receptor up-regulation considering the approx. 50% reduction of plasma cortisol levels achieved. Alternatively, receptors would remain

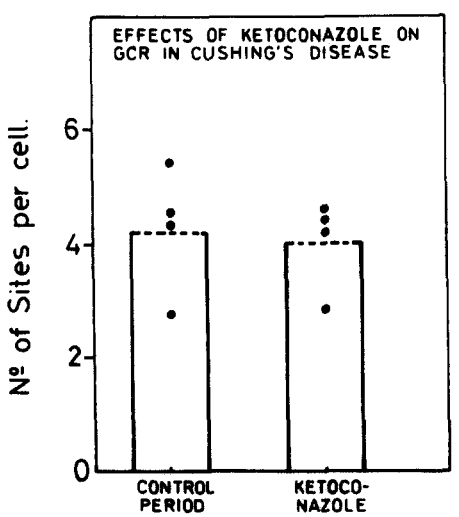

Fig. 4. Lack of effect of ketoconazole administered *in vivo* on GcR. Four patients with Cushing's disease received ketoconazole as indicated in the legend to Fig. 3. Each point indicates GcR in hMNL of an individual patient, with the group mean represented by the horizontal dashed line.

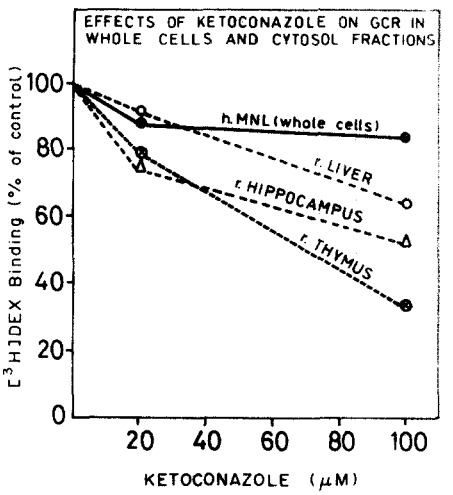

Fig. 5. Inhibition of GcR by ketoconazole added *in vitro*. GcR were determined in hMNL using a whole cell binding assay (——) or in cytosol from adrenalectomized rat liver, hippocampus and thymus (-----). Incubations contained [^{3}H]DEX alone or in combination with 20 or 100 μM ketoconazole. Results are expressed as % of the control value. Inhibition by 100 μM ketoconazole was significantly higher in the cytosol as compared to whole hMNL cell ($P < 0.02$).

unchanged if up-regulation was being counterbalanced by ketoconazoles antagonist action at the GcR level. To study this possibility, the antagonistic potency of ketoconazole was tested *in vitro*. However, addition of ketoconazole to hMNL cells incubated in the presence of [^{3}H]DEX effected a weak inhibition, amounting to $11.7 \pm 4.7\%$ at 20 μM and $13.6 \pm 4.5\%$ at 100 μM ($n = 3$, Fig. 5). This mild effect contrasted with the potency of ketoconazole on the binding of [^{3}H]DEX to soluble cytoplasmic receptors from rat hippocampus, thymus and liver (Fig. 5), in which case 20 μM effected a $17.1 \pm 2.7\%$ and 100 μM reached $53 \pm 7.4\%$ inhibition ($P < 0.02$). Therefore, ketoconazole antagonism applies to soluble receptors, but it was not obtained in intact cell preparations.

DISCUSSION

The present investigations have demonstrated that GcR in hMNL from patients receiving therapeutic doses of a synthetic glucocorticoid were down-regulated. In contrast, hypercortisolemic patients with pituitary-dependent Cushing's syndrome presented normal receptor levels, although a mild down-regulation emerged after the plasma cortisol concentration was contrasted with the receptor level of each individual patient. It is possible, therefore, that

GcR of hMNL are modulated not only by synthetic but also by natural ligands. However, the degree of down-regulation seems to depend on the biopotency of the glucocorticoid presented to the GcR, as exemplified for the AtT-20 cell GcR. In this case, DEX showed a 4-fold higher potency than corticosterone, a physiological adrenal secretory product [3].

Therefore, down-regulation seems a common phenomenon pertaining to GcR from different tissues and animal species [1–6] and present to limited amounts in Cushing's disease. In this group of patients, the slightly reduced GcR population showing normal affinity [9], would be sufficient to allow expression of the syndrome of cortisol excess. Contrarily, in patients with primary cortisol resistance, reduced affinity for [^{3}H]DEX in hMNL and reduced receptor number in cytosolic binding assays are correlated with lack of stigmata of Cushing's syndrome [17]. Although these observations suggest that glucocorticoid sensitivity could be assessed by determination of glucocorticoid binding to hMNL, there are numerous reports in the literature considering that the presence of GcR does not by itself guarantee steroid responsiveness, and that additional factors working at the physicochemical state of the receptor or at the genomic level would also determine the sensitivity of cells to hormonal action [18–20].

The ketoconazole studies provided some interesting observations for the clinical point of view. This drug was not a potent glucocorticoid antagonist when incubated with intact cells in this as well as in another study [21].

On the other hand, ketoconazole effectively displaced ligand binding from soluble cytosolic receptors such as DEX in hepatoma cells and triamcinolone acetonide in CEM-C7 leukemic cells [12, 21]. As shown in this communication, ketoconazole at 100 μM yielded approx. 50% inhibition of [^{3}H]DEX binding in cytosol of the hippocampus, thymus and liver, in comparison with a bare 13% reduction in whole hMNL. These data would therefore indicate that ketoconazole might not have access to GcR on intact hMNL or CEM-7 cells [21]. Alternatively, it is feasible that the second allosteric site of the GcR to which ketoconazole bind *in vitro* [22] promoting the release of the hormone from the steroid binding site of the receptor, is not available in intact cells. If ketoconazole cannot interact with GcR *in vivo*, as also suggested by results of GcR determination in hMNL prepared from Cushing's disease patients, it should be considered that the clincial improvement promoted by ketoconazole is caused by different mechanisms. In this regard, it is known ketoconazole inhibits cytochrome *P*450-dependent hydroxylases of cortisol biosynthesis in the adrenal gland [12], and that it also inhibits ACTH synthesis and secretion by a direct action at the anterior pituitary [23, 24]. The therapeutic value of ketoconazole would rather depend on these mechanisms and not on its antagonistic activity at the GcR level.

It should also be mentioned that after cortisol levels were reduced by ketoconazole treatment of patients with Cushing's disease, there was no evidence of GcR up-regulation in hMNL. Similarly, in patients with low cortisol levels due to Addison's disease studied by Schlechte and Sherman [11] and Kontula *et al.* [8], fewer or unchanged GcR were found. These authors concluded that changes in circulating adrenal hormone levels did not result in major alterations in GcR levels. Up-regulation, on the contrary was detected in human leukemic cells lines exposed to DEX [25]. These variable results, coupled to the mild down-regulation found in Cushing's disease, suggest that GcR control in humans may be a more complex phenomenon than that expected from animal studies in which overt negative and positive control of GcR followed increased or decreased availability of the plasma levels of the regulatory hormones. In cultured cells and animal tissues, two processes contribute to GcR regulation: first, an action on the receptor gene transcription, and second, changes in the half-life of the receptor protein [4, 26, 27]. Whether similar or different processes operate in hMNL, or if receptors in human pathologies are refractory to regulatory control by the ligand, is unknown and awaits further elucidation.

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