In vitro effects of ketoconazole on corticotrope cell morphology and ACTH secretion of two pituitary adenomas removed from patients with Nelson’s syndrome

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Abstract. The direct effects of ketoconazole on the secretion of ACTH by human pituitary adenoma cells from 2 patients with Nelson's syndrome were studied in vitro. Stereologically quantified, intracellular changes affect the surface density of the endoplasmic reticulum (it decreased by 73%), the volume density of the secretion granules (it decreased by 49%), and the volume density of lysosomes (it decreased by 67%). The hormone released in the culture medium decreased depending on the doses of ketoconazole used; 10 μmol/l decreased ACTH levels by 31%. These data show that ketoconazole induce marked changes on corticotrope morphology and ACTH secretion in pituitary cells obtained from patients with Nelson's syndrome.

Ketoconazole, an imidazole derivative used for its antimycotic action, inhibits ergosterol synthesis in fungi. In humans, as well as inhibiting cholesterol synthesis, it inhibits steroid synthesis in the suprarenal gland (glucocorticoids, mineralocorticoids and androgens) (1) and in the gonads (androgens and estrogens) (2) by inhibition of cytochrome P-450 dependent enzymes (11β-hydroxylase, 17-20 lyase, 20-22 desmolase, etc) (3).

In Cushing’s syndrome, ketoconazole has proved to normalize glucocorticoid function, both in short-term and in chronic treatments (4). A compensatory increase in the level of ACTH has also been reported throughout chronic treatment of Cushing’s syndrome with ketoconazole (5). There is also evidence of a decrease of ACTH, as well as of cortisol, during long-term treatment of Cushing’s syndrome (6).

In vitro studies with rat pituitary cells and human pituitary adenoma cells show that ketoconazole has no direct effects on the secretion of ACTH (7,8). However, Stalla et al. (9,10) and Julesz et al. (11) have reported a decrease of the release and biosynthesis of ACTH in rat pituitary cells.

This apparent contradiction as to increased or decreased ACTH levels during treatment of patients with Cushing’s syndrome has lead us to study in vitro effects of ketoconazole on ACTH-secreting pituitary cells from 2 patients with Nelson’s syndrome.

Patients and Methods

Patients
Patient No. 1: Female aged 35, with bilateral suprarenallectomy for Cushing’s disease. She had received corticoid treatment for 8 years. She presented extensive hyperpigmentation of the skin. Computed tomography (CT) revealed a 18-mm diameter tumour mass. ACTH serum level were 440 ng/l.
Patient No. 2: Female aged 23, with bilateral suprarenalectomy for Cushing’s disease. She had received corticoid treatment for 3 years. She presented extensive hyperpigmentation of the skin. CT revealed a 20-mm diameter tumour mass. ACTH serum level were 322 ng/l.

Preparation for immunohistochemical staining
Two adenomectomy specimens were obtained transspenoidally. A portion of tumour mass was fixed in 10% formalin for three days at 23°C. They were embedded in paraffin and cut into 2.5 µm thick sections. After deparaffini-

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Control</th>
<th>VvNu</th>
<th>VvCi</th>
<th>Vvgr</th>
<th>Vvmit</th>
<th>Vvly</th>
<th>SvRER</th>
<th>SvGo</th>
<th>Svmit</th>
<th>Hormonal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µmol/l KC</td>
<td>28.2 ± 2.3</td>
<td>49.3 ± 3.2</td>
<td>0.43 ± 0.08</td>
<td>0.93 ± 0.10</td>
<td>0.12 ± 0.02</td>
<td>32.2 ± 7.4</td>
<td>65.6 ± 10.0</td>
<td>54.6 ± 4.4</td>
<td>890 ± 161</td>
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</tr>
<tr>
<td>10 µmol/l KC</td>
<td>26.5 ± 3.2</td>
<td>51.4 ± 5.1</td>
<td>0.25 ± 0.10</td>
<td>0.81 ± 0.07</td>
<td>0.037 ± 0.01*</td>
<td>22.1 ± 4.5</td>
<td>43.0 ± 8.8</td>
<td>31.3 ± 4.6</td>
<td>706 ± 144</td>
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</table>

<table>
<thead>
<tr>
<th>Case 2</th>
<th>Control</th>
<th>VvNu</th>
<th>VvCi</th>
<th>Vvgr</th>
<th>Vvmit</th>
<th>Vvly</th>
<th>SvRER</th>
<th>SvGo</th>
<th>Svmit</th>
<th>Hormonal values</th>
</tr>
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<tbody>
<tr>
<td>2 µmol/l KC</td>
<td>23.4 ± 3.3</td>
<td>34.7 ± 5.3</td>
<td>0.43 ± 0.12</td>
<td>0.70 ± 0.10</td>
<td>1.12 ± 0.03</td>
<td>42.3 ± 13.7</td>
<td>33.2 ± 10.8</td>
<td>35.9 ± 10.8</td>
<td>850 ± 169</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1.
zation, they were treated with DAKO PAP™ Kit (DAKO-PATTS, Denmark) to stain human ACTH, GH and PRL (code No. K 514) and human FSH, LH and TSH (code No. K 517).

**Culture method**

The cellular dispersion was performed as previously described (12), except that 0.01 g/l collagenase (Type V, Sigma Chemical Co) was used for dispersion. Dispersed cells were plated onto plastic multivell culture dishes (5 × 10⁵ cells·ml⁻¹·well⁻¹) in Eagle’s medium, Earle’s BSS (Difco Laboratories, Detroit), pH 7.3, supplemented with 5% glutamine (Difco Laboratories), 10% fetal calf serum (Difco Laboratories), 0.1 IU/l of penicillin and 100 mg/l of streptomycin. Cultures were maintained in a CO₂ incubator (Flow Laboratories, Ayrshire, Scotland) at 37°C in humidified atmosphere of 98% air—2% CO₂.

Following 48 h of culture, the monolayer cultures were renewed with fresh serum-free medium adding 1 μmol/l and 10 μmol/l doses of ketoconazole (Janssen Pharmaceutical Ltd) to patient No. 1, 2 μmol/l of ketoconazole to patient No. 2, and serum-free medium without ketoconazole for control culture in both of them. Eight hours later, cultures were washed with phosphate buffered saline (PBS) and cells were removed by trypsinization (0.02 g/l for 10 min). Cells were transferred to Beem capsules (Agar, Essex, UK) and centrifuged at 100 × g for 10 min. The cellular pellets were fixed in 2% glutaraldehyde (Merck, FRG) and postfixed in 1% osmium tetroxide (Sigma Chemical Co). Following dehydration with ethanol they were embedded in Epon 812 (Polaron Equipment Ltd, Watford, UK).

**Morphological methods**

Ultrathin sections were taken from the resulting block to ultrastructural and stereologic study. They were stained with uranyl acetate and lead citrate and studied in a Jeol CX-200 microscope (Jeol Ltd, Tokyo). Sampling for stereologic analysis was randomly performed, considering the sample satisfactory only when standard error was lower than 10% of the mean. Micrographs were projected on different test systems (13). Volume and surface densities of the cytoplasmic organelles were calculated in relation to cytoplasmic volume density. Stereologic values were expressed in per cent (mean ± SD of 3 determinations). Test W of Shapiro & Wilk (14) was submitted to variance test and subsequently to Tukey’s HSD test (Q test) (15) with 95% and 90% significance level.

![Fig. 2](image)

ACTH-secreting pituitary adenoma cells of control culture (patient No.1). Ergastoplasm with evident cisternae. × 24 000.
ACTH-secreting pituitary adenoma cell of culture treated with 10 µmol/l ketoconazole (patient No. 1). Small cisternae of ergastoplasm. × 24 000.

Radioimmunoassay
Culture media ACTH was measured by radioimmunoassay using standard double-antibody technique. Antiserum was directed against human ACTH (Diagnostic Products Corporation).

Results
In both patients, immunohistochemical staining was positive only against ACTH.

Ketoconazole and cytoplasmic organelles
The effect of ketoconazole on different organelles of ACTH-producing cells from 2 patients with Nelson’s syndrome is shown in Fig. 1B–F. Fig. 1A shows the values of the different stereologic parameters studied.

In patient No. 1, depending on the dose we used, variations in the stereologic parameters of some cellular organelles were observed. With 1 µmol/l of ketoconazole only surface density of the endoplasmic reticulum decreased ($P < 0.05$). With 10 µmol/l of ketoconazole there was a decrease of volume density of secretion granules, of volume density of lysosomes ($P < 0.05$), of surface density of the endoplasmic reticulum ($P < 0.05$), and of surface density of mitochondria compared with control, whereas the decrease of surface density of the Golgi apparatus was not significant.

Ultrastructurally, corticotropic cells of control culture presented ergastoplasm with evident cisternae (Fig. 2). After 10 µmol/l of ketoconazole cisternae of ergastoplasm were less obvious in corticotropic cells (Fig. 3) than cisternae of control culture.

In patient No. 2, in whom only the dose of 2 µmol/l of ketoconazole was used there was a decrease of surface density of the endoplasmic reticulum compared with control.
ACTH release and ketoconazole

Data on the amount of hormone released are given in Fig. 1G. In both patients and with all doses used, there was a decrease of ACTH released into culture medium. In patient No. 1, the amount of ACTH in culture medium was 760 pg with 10 μmol/l of ketoconazole and 890 pg with 1 μmol/l. In patient No. 2, with 2 μmol/l of ketoconazole, the amount of ACTH in culture medium was 850 pg.

Discussion

Our results demonstrate that, in vitro, ketoconazole decreases ACTH secretion and release in pituitary adenoma cells from patients with Nelson’s syndrome.

The most remarkable aspect of our results is the considerable decrease of surface density of the endoplasmic reticulum (73% of control value) with 10 μmol/l of ketoconazole. This decrease can be correlated with the already proved inhibition by ketoconazole of cytochrome P-450 dependent enzymes (3). We believe that the decrease of volume density of secretion granules by 49%, compared with control culture, is the logical consequence of the decrease observed in surface density of the endoplasmic reticulum. It should be noted that the decrease of synthesis and release of ACTH, produced by ketoconazole, is accompanied by a decrease in volume density of lysosomes. All these data agree with an inhibition of ergastoplasm synthesis produced by ketoconazole.

The amount of ACTH released into culture medium through the cell monolayers of the pituitary adenoma cells from patients with Nelson’s syndrome is modified by ketoconazole. In patient No. 1, 10 μmol/l of ketoconazole decreased the ACTH release in culture medium by 31% compared with control, whereas with 1 μmol/l the decrease was only 20%. In patient No. 2, 2 μmol/l of ketoconazole decreased of the ACTH release in culture medium by 28% compared with control culture. Consequently, there seems to be a dose-dependent decrease, similar to the one described in rat corticotrophic cells (10), although there have also been reports on no direct effect being produced by ketoconazole on hormonal release in rats, both in vivo and in vitro (7).

In summary, our results show that in vitro treatment with ketoconazole of human pituitary adenoma cells derived from patients with Nelson’s syndrome induces marked morphological and functional changes in corticotrope cells. The biochemical and clinical significance thereof merits further study.

Acknowledgments

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References


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