Studies on glucocorticoid receptor gene polymorphism resulting in increased glucocorticoid sensitivity and on glucocorticoid overproduction in children

Abstract of Ph.D. thesis

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Budapest, 2007

Introduction

Glucocorticoids worldwide are one of the most frequently prescribed drugs. Because their therapeutic doses and the modes of their application are highly variable, it is of particular interest to obtain detailed information on the consequences of their long-term effects resulting in either a moderate or a substantial increase of glucocorticoid activity.

The pediatric patients enrolled in our study represent different types of glucocorticoid excess, which allowed us to examine the whole spectrum of the increased glucocorticoid effect.

The glucocorticoid receptor (GR) N363S polymorphism slightly increases the sensitivity to glucocorticoids and this slight alteration may exist from the beginning of the embryonic life. In our study the role of the sensitizing N363S polymorphism of the glucocorticoid receptor gene was examined in patients with congenital adrenal hyperplasia (CAH) and the results were correlated with clinical and hormonal findings.

Childhood obesity has become a challanging health issue worldwide. Glucocorticoids may have a role in the development of obesity or they may act as modifying factors in the metabolic consequences of the obesity. The N363S polymorphism has been reportedly associated with increased BMI in elderly patients. Therefore, we examined the allelic and carrier frequencies of the N363S polymorphism in obese children and compared the metabolic parameters of the N363S carrier and non-carrier groups. Glucocorticoid overproduction due to a cortisol-producing adrenal disorder or ectopic CRH/ACTH overproduction causes a dramatic increase of glucocorticoid activity, which is markedly different from that seen in cases with senzitizing polymorphism of the glucocorticoid receptor gene. We examined the effect of largely increased glucocorticoid production in two rare pediatric cases of Cushing's syndrome: in ectopic CRH/ACTH production by pancreatic acinar cell carcinoma and in primary pigmented nodular adrenocortical disease (PPNAD).

Our studies indicate that even a slight increase of glucocorticoid sensitivity may cause important physiological effects. The findings raise the necessity of further examinations whether a mild but prolonged increase of glucocorticoid activity such as exposure to local glucocorticoid therapy in children could exert unforeseen consequences. It is presumed that a better understanding of altered glucocorticoid sensitivity may help to develop individualized therapeutic regimens for our patients.

Our studies on glucocorticoid overproduction in children firmly indicate that despite characteristic clinical signs, the diagnosis of Cushing's syndrome is often difficult. The

prognosis depends on the early and precise diagnosis, the histological background, and the severity of clinical signs and complications.

Aims

My aim was to answer to the following questions

1. What is the allele frequency of the N363S polymorphism of the glucocorticoid receptor gene in CAH patients?
2. Is there any difference in the allele frequency of the N363S polymorphism of the glucocorticoid receptor gene between diferent subtypes of CAH?
3. Does the N363S polymorphism of the glucocorticoid receptor gene exert any effect on the phenotype of CAH patients?
4. Does the N363S carrier status influence the time of the diagnosis, the follow up or the somatic development of CAH patients?
5. What is the allele frequency of the N363S polymorphism of the glucocorticoid receptor gene in obese children?
6. Is there any difference in anthropometric data or in metabolic parameters between N363S carriers and non-carriers in a group of obese children?
7. What are the characteristic features of the overt shortterm glucocorticoid overproduction (caused by ectopic CRH/ACTH production) and the overt longterm glucocorticoid excess (caused by PPNAD)?

Patients and methods

Studies on the N363S polymorphism of the glucocorticoid receptor gene in CAH patients

Two hundred pediatric CAH patients were involved. The diagnosis was established by clinical signs, hormonal and genetic testing. There were 72 boys (46 salt-wasting [SW], 16 simple virilizing [SV] and 10 non-classical [NC]-CAH) and 128 girls (66 SW, 39SV, and 23 NC-CAH). In phenotype analysis twenty N363S non-carrier CAH patients were matched, as far as possible, to gender and the CYP21 allele class of the carriers. The severity of mutations was established according to the approach of Wedell et al.

The case histories were analyzed retrospectively. Anthropometric data of patients were compared to national standards. The bone age was determined according to Tanner-Whitehouse II.

The CYP21 genotype was analyzed by allele-specific PCR.

17-hydroxyprogesterone was measured by RIA.

Studies on the N363S polymorphism of the glucocorticoid receptor gene in obese children

Hundred obese children (51 girls, 49 boys; aged 3,8-18,1 years, age median: 12,7 years) were included.

After examining the frequency of the N363S polymorphism, the anthropometric data and metabolic parameters of carriers and non-carriers were compared.

The N363S polymorphism of the glucocorticoid receptor gene was determined by restriction fragment length polymorphism.

Serum electrolytes, liver enzymes, kidney function, thyroid hormone levels, glucose, total cholesterol, triglycerides and uric acid were measured using standard laboratory methods. Oral glucose tolerance test (OGTT) was performed according to WHO. Glucocorticoid overproduction was excluded by overnight dexamethasone test and 24- h urinary free cortisol measurement. Blood pressure was measured by ABPM.

Statistical analysis was performed by Statistica 6,0. Allele frequencies were compared by chi-square test or Fisher's exact test. The data of carriers and non-carriers were compared by ANOVA test, and Bonferoni posttest was also used.

Studies on glucocorticoid overproduction in pediatric patients with Cushing's syndrome

Primary pigmented nodular adrenocortical disease

The patient was a 12-year-old girl, who presented with hypertension, back pain, walking difficulties and Th.XII vertebral fracture was referred because of the suspicion of Cushing's syndrome.

Serum electrolytes, liver enzymes, kidney function, and glucose were measured using standard laboratory methods. Blood pressure was measured by ABPM.

The longterm low-dose dexamethasone suppression test was performed using a daily dose of 4 x 0,5 mg dexamethasone for two days, and the longterm high-dose dexamethasone was performed by the administration of a daily dose of 4 x 2,0 mg dexamethasone for three days.

The histological sections were stained with hematoxylin-eosin.

CRH/ACTH producing pancreas acinar cell carcinoma

The patient was a 9,5-year-old boy, who presented with back pain and hypertension. On examination an abdominal mass was found.

Serum electrolytes, liver enzymes, kidney function and glucose were measured using standard laboratory methods. Blood pressure was measured by ABPM.

Histological sections were stained with hematoxylin-eosin, and periodic acid Schiff. For immunhistochemistry mouse monoclonal and rabbit polyclonal antibodies were used. For electron microscopy tissue pieces were fixed by 2,5% glutaraldehyde and osmium tetroxide. For direct determination of the ACTH content of the tumor homogenates, an immunchemiluminometric assay was used.

Results

Studies on the N363S polymorphism of the glucocorticoid receptor gene in CAH patients

Of the 200 patients with CAH, 12 patients carried the N363S polymorphism (6%, 6 boys and 6 girls). When classified into subtypes, all the N363S carriers had classical CAH; 8 of the 12 carriers were SW, four had SV and none of them had NC-CAH. In the whole study population 17% had NC-CAH (33/200) and 83% had classical CAH.

The predicted phenotype regarding the CAH subtype based on CYP21 mutation analysis was different from the clinical phenotype in three carriers.

Since the frequency of the N363S polymorphism was higher in classical than in non-classical CAH (7,2% vs. 0), it seems likely that the N363S carrier status had an effect on the clinical severity of CAH.

Compared to the matched controls, the most important difference was that the N363S carrier girls tended to have less severely virilized genitalia at birth. There was no significant difference between carriers and non-carriers in terms of age at diagnosis (carriers 2 months [1 day-10 years]); non-carriers 1 month [2 days-5,5 years]). At the time of diagnosis 17-OHP levels were in the pathological range in all patients. The N363S carriers had somewhat lower 17-OHP levels than non-carriers (366 ± 283 ng/ml vs. 822 ± 727 ng/ml, P=0,09). The ratio of SW patients was almost identical in the carrier (8/12) and non-carrier (15/20) group (P=0,46).

There was no demonstrable difference in the growth pattern or required glucocorticoid dose between the carrier and non-carrier group.

As shown in earlier studies, glucocorticoid treated CAH patients are often obese. In the present study the prevalence of marked obesity defined by a body mass index (BMI) above 97th percentile for age was the same in the N363S carrier and non-carrier groups (6/20

and 4/12). The average age at the onset of obesity was higher in the N363S carrier group (median 7 years vs. 3,5 years).

Studies on the N363S polymorphism of the glucocorticoid receptor gene in obese children

Of the 100 obese children, 9 carried the N363S polymorphism (9%, 4boys and 5 girls).

There was no demonstrable difference in the frequency of low birth weight (< 2500 g) between the N363S carriers and non-carriers (1/6 vs. 5/81 *P*=0,35).

The frequency of obesity or signs of the metabolic syndrome in the family history was almost identical in the N363S carrier and non-carrier groups (9/9 vs. 72/79).

Of the 9 N363S carriers 2 children, and among the 91 non-carriers 17 children were obese since their birth. The difference was not significant. The average age at the onset of obesity was similar in both groups (carriers: 4 years, non-carriers. 3 years).

Of the 9 N363S carriers 2 children and among the 91 non-carriers 17 children had a weight-to-height above the 97th percentile. The difference was not significant.

There were no differences in weight SD, height SD and in BMI SD between the N363S carrier and non-carrier groups.

The frequency of overt obesity (BMI>95th percentile) was the same in both groups.

There were no significant differences in parameters of the carbohydrate metabolism between the N363S carriers and non-carriers. The frequency of fasting hyperinsulinaemia was the same in the two groups. The N363S carrier boys tended to have a higher HOMA index, and half of them had IGT. I could not find similar tendency among the N363S carrier girls.

The serum triglyceride, cholesterol and uric acid levels were similar in both groups.

The mean systolic and diastolic blood pressure was also the same in both groups.

Carriers tended to have lower frequency of hypertriglyceridemia, hyperuricaemia and hypertension. However. these differences did not reach the level of statistical significance.

Studies on glucocorticoid overproduction in children with Cushing' syndrome

Primary pigmented nodular adrenocortical disease

On admission the patient was short (height: 136,5 cm, <3rd percentile, -2,5SD), she had central obesity (bw: 55,1 kg, weight to height> 97th percentile) „ moon facies", striae and buffalo hump.

Laboratory findings indicated polyglobulia (Hgb163g/l), leucocytosis (15,8 G/l), hypokalemia (2.4 mmol/l), hyperglycemia (postprandial glucose 10.2 mmol/l), and glucosuria. Serum cortisol level was high without diurnal rhythm, and plasma ACTH was suppressed. Neither longterm low, nor longterm high dose of dexamethasone could suppress the serum cortisol level. These results confirmed an adrenal autonomous cortisol overproduction. Abdominal CT scan showed left adrenocortical hyperplasia.

The left adrenal gland was removed by laparoscopic adrenalectomy. The histological diagnosis was PPNAD. The postoperative period was uneventful. Eight months after the first operation she had an initially periodic laboratory (increased urinary free cortisol excretion) and clinical signs of cortisol excess (hypertension, hypokalemia). One year after the first operation the right adrenal gland was removed. The histology confirmed the first diagnosis. After the second operation the patient received glucocorticoid and mineralocorticoid supplementation. Since then she is in a good clinical condition. However, antihypertensive therapy could not be suspended because of the permanent hypertension.

CRH/ACTH producing pancreas acinar cell carcinoma

On admission the boy had normal height and weight (height: 135,5 cm, percentile: 25-50; body weight: 28,9 kg, percentile.25 - 50), „moon face", thin extremities, and enlarged abdomen. An overt muscular weakness was also present. Laboratory findings indicated hypokalemia (3.0 mmol/l) and hyperglycaemia (12.9-15.2mmol/l).

Abdominal ultrasound and CT scan showed an extended retroperitoneal mass (16x10x8 cm).

Among the tumor markers serum AFP was markedly increased (7664 IU/ml; normal, < 7.7), while serum NSE and βHCG were within the normal range.

Plasma ACTH level (08:00 a.m. 303.6 pg/ml; normal, 20-70), serum cortisol level (08:00 a.m. 44.1 μg/dl, normal: 8-25) and urinary free cortisol excretion (24-hour urine free cortisol 990 μg/g creatinine, normal: 50-200) were highly increased. Pituitary MRI was normal. All these findings were suggestive for an ectopic ACTH producing tumor.

Explorative laparotomy revealed the bulky tumor mass appearing to arise from the body of the pancreas with anterior displacement of the stomach. The tumor invaded around of the hilum of the left kidney and the portal and the inferior caval vein. Due to the involvement of these vessels, the tumor was considered inoperable and tumor samples were obtained for

histology. Histological examination indicated an ectopic CRH/ACTH producing pancreas acinar cell carcinoma.

After nine blocks of chemotherapy, radiotherapy (30 Gy) was applied. The tumor became smaller but remained inoperable.

After three months of treatment the patient was normotensive, normokalemic and euglycaemic without any treatment. Serum ACTH and serum cortisol definitely normalized, and serum AFP lowered significantly. After six months serum AFP level increased again, and lung metastases was detected without any biochemical or clinical evidence for ectopic hormone production. Six months later the child died because of the tumor progression.

Conclusions

1. We found that the allele frequency of the N363S polymorphism of the glucocorticoid receptor gene in CAH patients was 6%, which was similar to that found in healthy Hungarian population (7,8%).
2. Among the N363S carrier CAH patient no patients with NC-CAH were found, while in the whole study population NC-CAH accounted for 17% of all CAH patients. We can hypothesize that The N363S carrier status may decrease the signs of hyperandrogenism in the affected patients.
3. The N363S carrier girls were less severely virilized than matched non-carriers. It could be the consequence of lower androgen effect in the early intrauterine life.
4. There were no differences in the age of the diagnosis, the growth pattern and the required glucocorticoid doses between carriers and non-carriers. The frequency of obesity was the same in the carrier and non-carrier groups but the onset of obesity was later in the N363S carriers and seemed to be progressive during puberty.
5. The frequency of the N363S polymorphism was 9% in obese children. This allelic frequency failed to differ from that reported in Hungarian healthy population.
6. There were no differences between the N363S carrier and non-carrier groups of obese children in terms of gender and ageThe anthropometric parameters were also the same in the N363S carrier and non-carrier groups. N363S carrier boys tended to have more disturbances in carbohydrate metabolism (IGT) than non-carriers, but the difference was not significant. The frequency of hypertriglyeridemia, hyperuricaemia and hypertension were lower in the N363S carriers. However, the difference was not statistically significant.

7. In children the shortterm glucocorticoid overproduction may present with normal height and weight, „moon faciei", thin extremities, reversible hyperglycemia and hypokalemia. In contrast, longterm cortisol overproduction causes obesity, short stature, overt left ventricular hypertrophy and osteoporosis. In the latter case hyperglycemia was reversible, but hypertension persisted and needed antihypertensive treatment.

Publications related to the theses

Original articles:

1. **Luczay A,** Török D, Ferenczi A, Majnik J, Sólyom J, Fekete Gy (2006) Potential advantage of N363S glucocorticoid receptor polymorphism in 21-hydroxylase deficiency Eur J Endocrinol, 154 : 859-864 **IF: 3,072**

2. Illyés G, **Luczay A,** Benyó G, Kálmán A, Borka K, Köves K, Rácz K, Tulassay T, Schaff Z (2007) Cushing's syndrome in a child with pancreatic acinar cell carcinoma. Endocr Pathol 18:95-102 **IF***:**1,50**

3. **Luczay A.,** Ferenczi A., Török D., Majnik J., Sólyom J., Fekete Gy (2006) A GR gén N363S polymorphizmusának vizsgálata congenitalis adrenalis hyperplasia, 21-hidroxiláz defektus formájában szenvedő betegekben. Gyermekgyógyászat, 4:421-428

4. Majnik J., Patócs A., Balogh K., **Luczay A.,** Török D., Szabó V., Borgulya G., Gergics P., Szappanos Á., Bertalan R., Belema B., Tőke J., Sereg M., Nagy ZZ., Sólyom J., Tóth M., Gláz E., Rácz K., Németh J., Fekete Gy., Tulassay Zs. (2006) A glükokortikoid receptor variánsainak klinikai jelentősége. Orv Hetil. 147:2107-2115

Abstracts

1. **Luczay A,** Illés Gy, Kálmán A, Rényi I, Rácz K, Sólyom J, Fekete (2004) Cushing's syndrome due to corticotropin-relaesing hormone secreting pancreatoblastoma in a ten years old child. Horm Res 62(S2);94,

2. **Luczay A,** Török D, Ferenczi A, Battelino T, Majnik J, Sallai Á, Gács Zs, Sólyom J (2005) The N363S polymorphism of the glucocorticoid receptor modifies the phenotype of congenital adrenal hyperplasia. A pilot study. Horm Res 64 (S1);336
3. **Luczay A**, Török D, Ferenczi A, Majnik J, Battelino T, Sallai Á, Gács Zs, Sólyom J, Fekete Gy (2004) The N363S polymophismus of the glucocorticoid receptor: potential contribution to phenotype of CAH – case report
   Slov Ped 11(3)92
4. **Luczay A,** Török D, Ferenczi A, Halász Z, Hosszú É, Sallai Á, Sólyom J, Fekete Gy (2005) The N363S polymorphism of the glucocorticoid receptor in obese children Diabet. Metab. Endocrin. Vyziva 8 (S2) 16,

Publications not directly related to the theses

Original articles:

1. **Luczay A,** Vásárhelyi B, Dobos M, Holics K, Újhelyi R, Tulassay T (1997) Altered erythrocyte sodium-lithium counter transport and Na^+ / K^+-ATP-ase activity in cystic fibrosis Acta Pediatr 86;245-7 **IF:0,81**
2. Tulassay T, Dobos M, **Luczay A,** Stubnya G, Reusz G.S., Vásárhelyi B, Sallay P, Szabó A (1999) Sodium-lithium countertransport in children with nephrotic syndrome Pediatr Nephrol 13; 510-13 **IF:1,159**
3. **Luczay A.,** Vásárhelyi B., Madácsy L., Pánczél P, Tulassay T (2001) Szigetsejt-citoplazma és glutaminsav-dekarboxiláz elleni antitestek kis súllyal született fiatal felnőttekben Orv Hetilap 39;2145-2147
4. **Luczay A** (2003) A növekedési hormon kezelés lehetőségei juvenilis rheumatoid arthritisben (irodalmi áttekintés) Gyermekgyógyászat, 4;472-476
5. **Luczay A,** Sólyom J (2006) Alacsony SGA (small for gestational age) gyermekek növekedési hormon kezeléséről Gyermekgyógyászat, 6:721-724
6. **Luczay A,** Sólyom J, Hiort O, Szabó E, Dobos M, Jenővári Z. Fekete Gy (2006) Inkomplett androgén inszenzitivitás szindróma Orv Hetil. 147:1805-1807.
7. Pánczél P, Külkey O, **Luczay A,** Bornemisza B, Illyés G, Halmos T, Baranyi É, Blatniczky L, Mészáros J, Kerényi Zs, Gerő L, Tamás Gy, Hosszúfalusi N, Horváth L,

Madácsy L, Romics L(1999) Hasnyálmirigy-szigetsejt elleni antitestek a klinikai gyakorlatban Orv Hetil. 140:2695-2701

8. Krikovszky D, **Luczay A,** Körner A, Madácsy L (2000) Gyermekkori diabeteses ketoacidózis Orv Hetil 141:173-177

9**.** Pánczél P, Hosszúfalusi N, Bornemisza B, Horváth L, Jánoskúti L, Füst G, Rajczy K,Vatay Á, Prohászka Y, Madácsy L, Luczay A, Blatniczky L, Halmos T, Körner A, Szilvási I, Romics L(2001) A lassan kialakuló felnőttkori autoimmun diabetes (LADA): az 1-es típusú cukorbetegség spektrumának része. Orv Hetil. 142 :2571-2578

10. Cserbák A, **Luczay A,** Szabó M, Machay T (2006) Neurogén respirációs distress Gyermekgyógyászat 2;136-140,