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The Response of C_{19} Δ^5-steroids to ACTH Stimulation and Hypoglycemia in Insulin Tolerance Test for Adrenal Insufficiency

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Abstract: Studies on the time course of ACTH- or insulin-induced hypoglycemia stimulating adrenal androgens are usually limited to dehydroepiandrosterone and/or its sulphate. Our data on dehydroepiandrosterone (DHEA) and its hydroxylated metabolites clearly show that measurements of DHEA and its sulphate (DHEAS) are valuable markers of the integrity of the HPA (hypothalamus-pituitary-adrenal) axis. Assessments of HPA function should rely on measurements of baseline and/or stimulated serum cortisol concentrations, and C_{19} Δ^5-steroids may provide additional information. The art of stimulation of 7- and 16-hydroxylated metabolites of DHEA can help our understanding of the formation sequence of these compounds.

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Introduction

The adrenal gland is a key provider of androgens in women (Short, 1960). The adrenal androgens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, 5-andostenediol and 11β-hydroxy-androstenedione exert only little androgenic activity; however, they provide a pool of circulating precursors for more active androgens or steroids, with a wide spectrum of important physiological effects. The role of DHEA and DHEAS as neurosteroids has been intensively studied (Baulieu and Robel, 1998; Morfin and Stárka, 2001; Labrie, 2010; Li and Bigelow, 2010; Hill et al., 2015; Stárka et al., 2015). Its 7-hydroxylated metabolites modulate our immunity (Pélissier et al., 2004, 2006; Stickney et al., 2011) and take part in the control of local cortisol levels through competition in the 11β-hydroxysteroid dehydrogenase system (Hennebert et al., 2007; Sedláčková et al., 2012). The 16-hydroxylated derivatives may play some role in hormonal homeostasis (Hampl and Stárka, 2000). In spite of the importance of C_{19} Δ^5-steroids, their responsiveness to corticotrophin or insulin-induced hypoglycemia has not been well studied (Rege et al., 2013). In the present study, we quantified ten C_{19} Δ^5-steroids plasma samples from women before and after ACTH stimulation, and compared the results with stimulation of the hypothalamus-pituitary-adrenal (HPA) system by insulin-induced hypoglycemia. Dynamic testing of adrenal function is a standard procedure for the diagnosis of adrenal insufficiency, and we tried to find differences in the ACTH stimulation test in terms of C_{19} Δ^5-steroids between healthy women and patients with adrenal insufficiency.

Material and Methods

Our study involved six premenopausal females (with mean/median 43.4/42 age (SD ±6.2) years, and mean/median 25.4/24.6 BMI (body mass index) (SD ±3.9) kg/m^2) with primary adrenal insufficiency verified by clinical symptoms and biochemical markers. The control group consisted of seven healthy BMI and age matched women. The women used no medications and had no history of using corticosteroids. All signed informed consent before initiating the study. The study was approved by the Ethical Commission of the Institute of Endocrinology.

All patients underwent the 250 μg "high dose" ACTH test. The control group was given two tests: the 250 μg "high dose" ACTH test and the insulin tolerance test (ITT). The minimum time between tests was one week. All tests were performed after an overnight fast, and started in the morning between 7 and 9 a.m. Synacthen and insulin were administered through a cannula inserted into the cubital vein, 15 minutes after insertion of the cannula.

Dynamic testing

Details on the dynamic testing have been presented elsewhere (Šimůnková et al., 2015; Dušková et al., 2016). The tests are described in brief as follows:

High dose ACTH stimulation (HDST): The contents of 1 ampule 250 μg/1 ml Synacthen (tetracosactide 250 μg, Novartis Pharma GmbH, Nuernberg, Germany) was given intravenously after first blood sample drawn (time = 0), and then at 30, 60, and 90 minutes.

Insulin-induced hypoglycemia stimulation – ITT: 0.1 IU per 1 kg Actrapid insulin was given intravenously. During the test, blood glucose was regularly checked with a glucometer (Accu-Chek Perform), and blood pressure and pulse rate were measured every five minutes during the first hour and every ten minutes thereafter. There was a decrease in blood glucose below 2.2 mmol/l in all of the tests, and all controls had a spontaneous blood glucose response during the first hour followed by normalization. Blood samples were taken prior to the administration of insulin at time = 0, and then after 20, 30, 40, 60, 90, and 120 minutes. The ITT was only carried out in the control group since it is unpleasant and may introduce risks in patients with adrenal insufficiency.

Analytical measurements

Steroid hormones measured by the GC/MS method

The levels of C_{19} Δ^5-steroids and of additional 27 unconjugated steroids and their polar conjugates were measured in cubital vein blood using our original GC/MS method (Hill et al., 2010). In brief, free steroids were extracted from plasma by diethyl-ether; steroid conjugates were then hydrolyzed and extracted. The resulting residues were derivatized by methoxyamine hydrochloride and analysed by GC/MS as described below.

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Instruments

Measurements of steroid levels were performed on a GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, an AOC-20s autosampler, and a single quadrupole detector with an adjustable electron voltage of 10–195 V. A capillary column with a medium polarity RESTEK Rxi phase (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron impact ionization with electron voltage fixed at 70 V and emission current set to 160 μA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode (200 kPa), which was maintained for 1 min. The detector voltage was set to 1.4 kV.

Statistical analysis

Data were transformed by Box-Cox transformation before further processing due to non-Gaussian distribution and non-constant variance (heteroscedasticity) in all variables. Repeated-measures analysis of variance (ANOVA) was used for monitoring levels of steroids during the tests. The ANOVA model was followed by least significant difference (LSD) multiple comparisons. The statistical software Statgraphics Centurion, version XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations and ANOVA analyses.

Results

Basal plasma levels of C_{19} Δ^5-steroids and their levels at 60 min after ACTH stimulation are given in Table 1, and for insulin-induced hypoglycemia in Table 2. The trends of the steroid levels after the stimulation of adrenal secretion by ACTH and by insulin-induced hypoglycemia are shown in Figures 1–5.

Table 1 – Baselines and values at 60th min of ACTH test for patients and controls

Steroid	ACTH test					
	Controls		change fold	Patients		change fold
	0 min	60th min		0 min	60th min	
dehydroepiandro-sterone	5.46 (4.22, 6.97)	19.2 (16.5, 22.4)	3.52*	4.81 (3.58, 6.37)	7.42 (5.91, 9.24)	1.54
conjugated DHEA	986 (893, 1080)	1020 (927, 1120)	1.30	441 (356, 537)	330 (271, 397)	0.75
7α-hydroxy-DHEA	0.53 (0.42, 0.66)	0.94 (0.75, 1.18)	1.78*	0.29 (0.20, 0.42)	0.40 (0.27, 0.57)	1.38
7β-hydroxy-DHEA	0.29 (0.24, 0.35)	0.31 (0.26, 0.38)	1.10	0.21 (0.15, 0.30)	0.18 (0.12, 0.25)	0.64
16α-hydroxy-DHEA	0.02 (0.02, 0.04)	0.03 (0.02, 0.04)	1.21	0.07 (0.04, 0.12)	0.04 (0.03, 0.07)	0.65
conjugated 16α-hydroxy-DHEA	2.95 (2.15, 3.88)	3.01 (2.2, 3.9)	1.10	0.57 (0.07, 1.35)	1.71 (0.95, 2.69)	3.70
5-androsten-3β, 17β-diol	0.62 (0.46, 0.89)	1.00 (0.77, 1.33)	1.60*	0.34 (0.24, 0.51)	0.25 (0.19, 0.32)	0.72
conjugated androstenediol	235 (214, 258)	219 (198, 241)	0.93	101 (81, 124)	69.70 (56.1, 85)	0.69
5-androstene-3β, 7α,17β-triol	0.11 (0.087, 0.133)	0.17 (0.13, 0.21)	1.55*	0.04 (0.03, 0.06)	0.05 (0.03, 0.06)	1.11
5-androstene-3β, 7β,17β-triol	0.08 (0.067, 0.105)	0.12 (0.09, 0.15)	1.40	0.06 (0.04, 0.08)	0.04 (0.031, 0.061)	0.72

Median and quartiles are given; *significance at $p<0.05$

Table 2 – Baselines and values at 60th min of ITT test for controls

Steroid	ITT test		
	Controls		change fold
	0 min	60th min	
dehydroepiandrosterone	6.69 (5.34, 8.41)	15.80 (12.6, 20)	2.36*
conjugated DHEA	1070 (899, 1260)	1090 (921, 1300)	1.20
7α-hydroxy-DHEA	0.43 (0.39, 0.48)	0.63 (0.557, 0.715)	1.46*
7β-hydroxy-DHEA	0.27 (0.226, 0.321)	0.33 (0.28, 0.39)	1.22
16α-hydroxy-DHEA	0.03 (0.013, 0.065)	0.02 (0.013, 0.039)	0.79
conjugated 16α-hydroxy-DHEA	2.12 (1.67, 2.63)	2.10 (1.66, 2.62)	0.99
5-androsten-3β,17β-diol	0.68 (0.57, 0.82)	0.85 (0.747, 0.97)	1.25*
conjugated 5-androstenediol	245 (236, 318)	269 (230, 312)	1.12
5-androsten-3β,7α,17β-triol	0.16 (0.088, 0.380)	0.08 (0.060, 0.117)	0.52
5-androsten-3β,7β,17β-triol	0.06 (0.041, 0.101)	0.07 (0.055, 0.098)	1.16

Median and quartiles are given; *significance at p<0.05

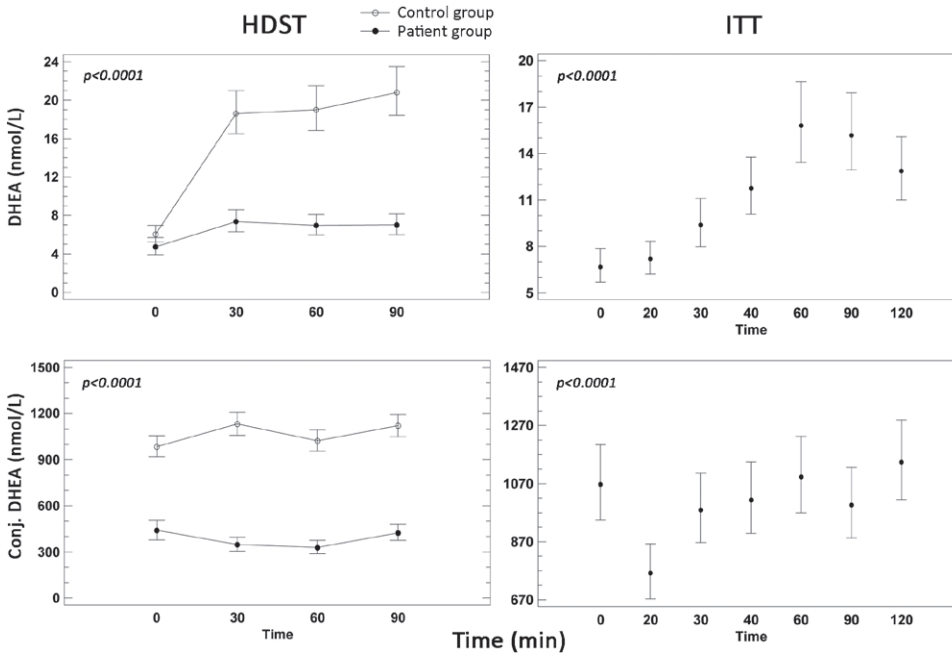

Figure 1 – Plasma dehydroepiandrosterone (DHEA) and conjugated DHEA in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

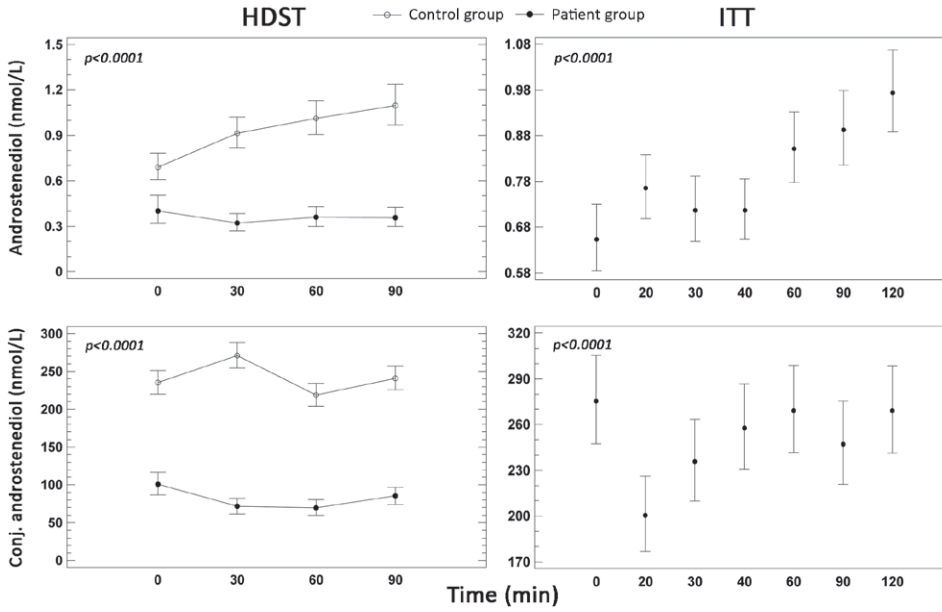

Figure 2 – Plasma 5-androstene-3β,17β-diol (androstenediol) and conjugated androstenediol in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

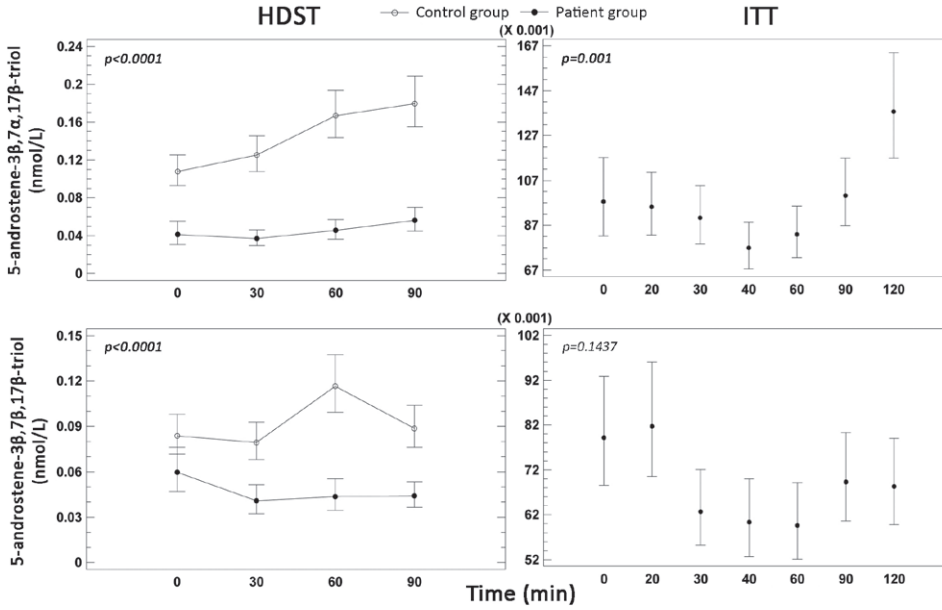

Figure 3 – Plasma 5-androstene-3β,7α,17β-triol and 5-andostene-3β,7β,17β-triol in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

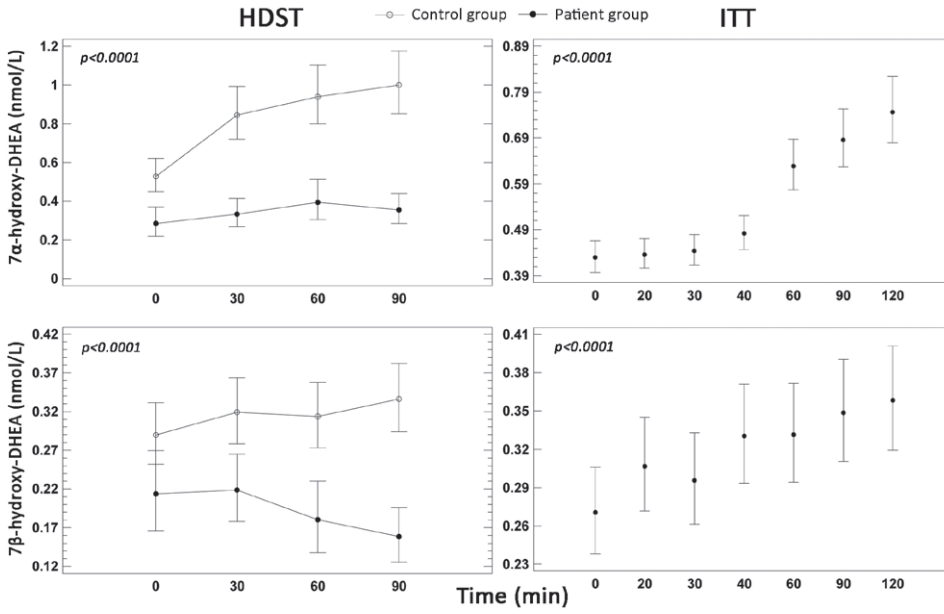

Figure 4 – Plasma 7α-hydroxy- and 7β-hydroxy-dehydroepiandrosterone in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

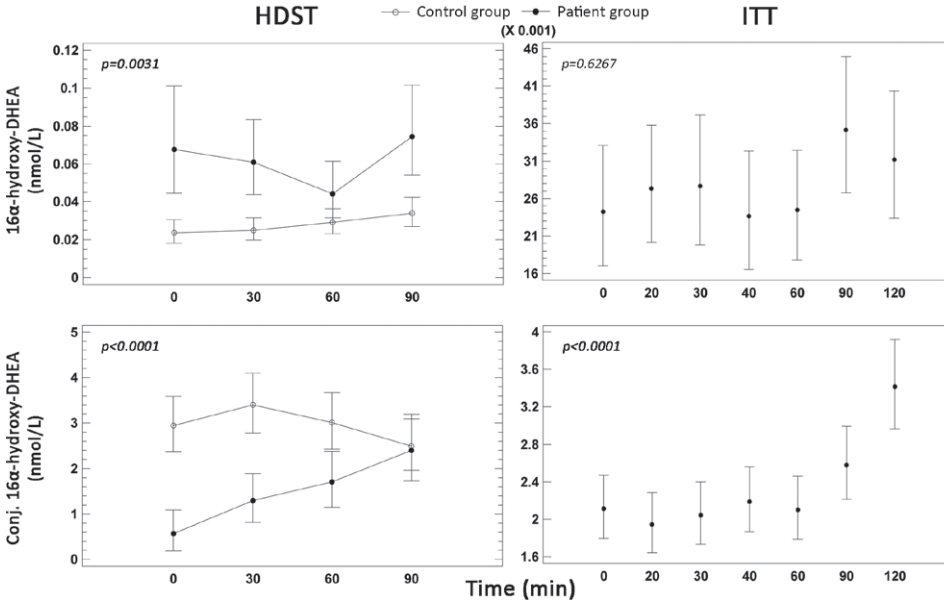

Figure 5 – Plasma 16α-hydroxy-DHEA and conjugated 16α-hydroxy-DHEA in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

1) For all steroids, both free and conjugated, levels during the ACTH tests in patients did not show significant increases, in contrast to the levels in healthy controls.
2) Except for free DHEA and 7β-hydroxylated derivatives of DHEA, even the starting levels of the steroids significantly differed between the patients with adrenal insufficiency and healthy controls.
3) In contrast to free steroids, changes of the levels of their conjugates during the ACTH test were insignificant.
4) During the ITT, the increase of steroid conjugates was also either insignificant, or in some rare cases, as for conjugated 16α-hydroxy-DHEA, the increase was delayed.
5) All free steroids (with the exception of 16α-hydroxy-DHEA) increased after both stimuli; however, the increase during the ITT had a latent time of about 20–40 min.
6) The differences between the baseline and peak concentration at 60 min of C_{19} Δ^5-steroids in healthy controls was higher during the ACTH test than during the ITT.

Discussion

Dynamic testing of adrenal function is a standard procedure for the diagnosis of proper adrenal function. Testing with various doses of ACTH-24 or with insulin-induced hypoglycemia (insulin tolerance test – ITT) is commonly used, and the concentration of circulating cortisol, either in plasma or in saliva, generally serves as the marker measured. In establishing the diagnosis of adrenal insufficiency, several authors have recommended measurements of baseline serum cortisol and DHEAS levels (Abdu et al., 1999; Al-Aridi et al., 2011). Our results indicated that it may be useful to additionally follow the stimulation of C_{19} Δ^5-steroids during the course of stimulation by either ACTH or ITT. Increases of DHEA or DHEAS after stimulation are observed in plasma but not in saliva (Dušková et al., 2016), due to the insufficient ultrafiltration of these compounds to saliva.

Our present data also show that the levels of C_{19} Δ^5-steroids are reliable markers of adrenal insufficiency. Even the basal levels of conjugated DHEA, free or conjugated 5-androstenediol, free 7α-hydroxy- and 16α-hydroxymetabolites of DHEA differed significantly between healthy subjects and patients with impaired adrenal function. In contrast to healthy individuals, there was no increase of C_{19} Δ^5-steroids during the ACTH test in the group of patients with adrenal insufficiency.

The lack of an increase of conjugated C_{19} Δ^5-steroids both during the ACTH and ITT tests before 60 min is in agreement with other findings of the postponed increase of conjugated DHEA (Sayyed Kassem et al., 2012), and with the observation that there is a stimulation of DHEA but no response in DHEAS production by ACTH in normal adrenocortical cell suspensions (Fehér et al., 1985). This is compatible with the differences in the half-times of DHEA (2–3 h) and

DHEAS (20 h) (Kroboth et al., 1999) and with the concept that the biosynthesis of DHEA is under the control of ACTH, while other factors may contribute to the regulation of the sulphate pathway of DHEA secretion under normal conditions.

The effective and rapid stimulation of adrenal C_{19} Δ^5-steroids by ACTH is an argument against the hypothesis of the presence of a special unknown hypothalamic or pituitary adrenal androgen-stimulating hormone (Parker and Odell, 1979; Odell and Parker, 1984–1985), a hypothesis that until now has neither been confirmed nor refuted.

Whereas changes of DHEA or DHEAS levels in the course of dynamic testing have been reported in many studies (Griffing et al., 1985; Abdu et al., 1999; Al-Aridi et al., 2011; Sayyed Kassem et al., 2012), the response of hydroxylated derivatives of DHEA had not yet been studied. 7α-hydroxymetabolites are rapidly stimulated by both ACTH and ITT in coordination with DHEA levels, while an increase of 7β-hydroxymetabolites is either delayed or absent. This is in agreement the concept that 7α-hydroxylation is the primary reaction and that 7β-epimers are a later product of either direct isomerization or conversion through a 7-oxo-derivative.

A quite exceptional reaction to stimulation was observed for 16α-hydroxy-DHEA. We found that the levels of free 16α-hydroxy-DHEA did not change significantly during either the ACTH or ITT tests; however, conjugated 16α-hydroxy-DHEA increased during the ACTH test in the group of adrenal insufficient women as well as in controls during the ITT.

In conclusion, our data clearly show that DHEA and DHEAS measurements are valuable markers of the integrity of the HPA axis. Assessments of HPA function should rely on the measurement of baseline and/or ACTH-stimulated serum cortisol concentrations, and C_{19} Δ^5-steroids may provide additional information. The art of stimulation of hydroxylated metabolites of DHEA can help improve our understanding of the sequence of the formation of these compounds.

References

Abdu, T. A., Elhadd, T. A., Neary, R., Clayton, R. N. (1999) Comparison of the low dose short synacthen test (1 microg), the conventional dose short synacthen test (250 microg), and the insulin tolerance test for assessment of the hypothalamo-pituitary-adrenal axis in patients with pituitary disease. *J. Clin. Endocrinol. Metab.* **84(3)**, 838–843.

Al-Aridi, R., Abdelmannan, D., Arafah, B. M. (2011) Biochemical diagnosis of adrenal insufficiency: the added value of dehydroepiandrosterone sulfate measurements. *Endocr. Pract.* **17(2)**, 261–270.

Baulieu, E. E., Robel, P. (1998) Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. *Proc. Natl. Acad. Sci. U. S. A.* **95(8)**, 4089–4091.

Dušková, M., Šimůnková, K., Vítků, J., Sosvorová, L., Jandíková, H., Pospíšilová, H., Šrámková, M., Kosák, M., Kršek, M., Hána, V., Žánová, M., Springer, D., Stárka, L. (2016) A comparison of salivary steroid levels during diagnostic tests for adrenal insufficiency. *Prague Med. Rep*. **117(1)**, 18–33.

Fehér, T., Szalay, K. S., Szilágyi, G. (1985) Effect of ACTH and prolactin on dehydroepiandrosterone, its sulfate ester and cortisol production by normal and tumorous human adrenocortical cells. *J. Steroid Biochem.* **23(2)**, 153–157.

Griffing, G. T., Allen, J., Pratt, H., Melby, J. C. (1985) Discordance of plasma DHEA-S, DHEA, and cortisol responses with various ACTH regimens. *Metabolism* **34(7)**, 631–636.

Hampl, R., Stárka, L. (2000) Minireview: 16alfa-hydroxylated metabolites of dehydroepiandrosterone and their biological significance. *Endocr. Regul.* **34(3)**, 161–163.

Hennebert, O., Chalbot, S., Alran, S., Morfin, R. (2007) Dehydroepiandrosterone 7alpha-hydroxylation in human tissues: possible interference with type 1 11beta-hydroxysteroid dehydrogenase-mediated processes. *J. Steroid Biochem. Mol. Biol.* **104(3–5)**, 326–333.

Hill, M., Pařízek, A., Cibula, D., Kancheva, R., Jirásek, J. E., Jirkovská, M., Velíková, M., Kubátová, J., Klímková, M., Pašková, A., Žižka, Z., Kancheva, L., Kazihnitková, H., Zamrazilová, L., Stárka, L. (2010) Steroid metabolome in fetal and maternal body fluids in human late pregnancy. *J. Steroid Biochem. Mol. Biol.* **122(4)**, 114–132.

Hill, M., Dušková, M., Stárka, L. (2015) Dehydroepiandrosterone, its metabolites and ion channels. *J. Steroid Biochem. Mol. Biol.* **145**, 293–314.

Kroboth, P. D., Salek, F. S., Pittenger, A. L., Fabian, T. J., Frye, R. F. (1999) DHEA and DHEA-S: a review. *J. Clin. Pharmacol.* **39**, 327–348.

Labrie, F. (2010) DHEA, important source of sex steroids in men and even more in women. *Prog. Brain Res.* **182**, 97–148.

Li, A., Bigelow, J. C. (2010) The 7-hydroxylation of dehydroepiandrosterone in rat brain. *Steroids* **75(6)**, 404–410.

Morfin, R., Stárka, L. (2001) Neurosteroid 7-hydroxylation products in the brain. *Int. Rev. Neurobiol.* **46**, 79–95.

Odell, W. D., Parker, L. N. (1984–1985) Control of adrenal androgen production. *Endocr. Res.* **10(3–4)**, 617–630.

Parker, L. N., Odell, W. D. (1979) Evidence for existence of cortical androgen-stimulating hormone. *Am. J. Physiol.* **236(6)**, E616–E620.

Pélissier, M. A., Trap, C., Malewiak, M. I., Morfin, R. (2004) Antioxidant effects of dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat colon, intestine and liver. *Steroids* **69(2)**, 137–144.

Pélissier, M. A., Muller, C., Hill, M., Morfin, R. (2006) Protection against dextran sodium sulfate-induced colitis by dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat. *Steroids* **71(3)**, 240–248.

Rege, J., Nakamura, Y., Satoh, F., Morimoto, R., Kennedy, M. R., Layman, L. C., Honma, S., Sasano, H., Rainey, W. E. (2013) Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. *J. Clin. Endocrinol. Metab.* **98(3)**, 1182–1188.

Sayyed Kassem, L., El Sibai, K., Chaiban, J., Abdelmannan, D., Arafah, B. M. (2012) Measurements of serum DHEA and DHEA sulphate levels improve the accuracy of the low-dose cosyntropin test in the diagnosis of central adrenal insufficiency. *J. Clin. Endocrinol. Metab.* **97(10)**, 3655–3662.

Sedláčková, B., Dušátková, L., Zamrazilová, H., Matucha, P., Bičíková, M., Stárka, L. (2012) 7-oxygenated derivatives of dehydroepiandrosterone and obesity. *Prague Med. Rep.* **113(2)**, 147–155.

Short, R. V. (1960) The secretion of sex hormones by the adrenal gland. *Biochem. Soc. Symp.* **18**, 59–84.

Šimůnková, K., Dušková, M., Kosák, M., Kršek, M., Hána, V., Hill, M., Jandíková, H., Pospíšilová, H., Šrámková, M., Bifulco, E., Stárka, L. (2015) Response of cortisol metabolites in the insulin tolerance test and Synacthen tests. *Physiol. Res.* **64**, S237–S246 (Suppl. 2).

Stárka, L., Dušková, M., Hill, M. (2015) Dehydroepiandrosterone as a neurosteroid. *J. Steroid Biochem. Mol. Biol.* **145**, 254–260.

Stickney, D. R., Ahlem, C. N., Morgan, E., Reading, C. L., Onizuka, N., Frincke, J. M. (2011) Phase I and Phase II clinical trials of androst-5-ene-3β,7β,17β-triol. *Am. J. Transl. Res.* **3(3)**, 275–283.