J Med Biochem 2011; 30 (4) DOI: 10.2478/v10011-011-0030-6

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 30: 287–292, 2011

Original paper
Originalni naučni rad

MORPHOFUNCTIONAL CHARACTERISTICS OF PITUITARY ADRENOCORTICOTROPES IN AN ANIMAL MODEL OF HEAT STRESS

MORFOFUNKCIONALNE OSOBINE HIPOFIZNIH ADRENOKORTIKOTROPA U ŽIVOTINJSKOM MODELU TOPLOTNOG STRESA

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Summary: As a result of the global warming, the average ambient temperature during summertime has increased in regions with moderate continental climate. The effects of 24 h exposure to heat stress (35±1 °C) on the morphology and function of pituitary adrenocorticotropes were examined in adult male Wistar rats. Significant changes in the morphofunctional features of adrenocorticotropes were found after the heat stress, with no differences noted in the cell shape or localization, compared to controls. The adrenocorticotropes cell volume, as well as the volume density, were significantly decreased ($p<0.05$) by 12.3% and 26.7%, respectively, in comparison with controls. The concentration of plasma adrenocorticotropic hormone and serum corticosterone in the heat stressed group were significantly decreased ($p<0.05$) by 21.9% and 27.2%, respectively, compared to controls. These findings suggest that 24 h exposure of adult male rats to heat stress has an inhibitory effect on the morphofunctional characteristics of adrenocorticotropes.

Keywords: heat stress, ACTH, male rats

Kratak sadržaj: Usled globalnog otopljavanja u regionima sa umereno kontinentalnom klimom dolazi do povećanja srednje vrednosti temperature vazduha tokom letnjih meseci. U ovoj studiji ispitivani su efekti 24-časovnog izlaganja toplotnom stresu (35±1 °C) na morfologiju i funkciju hipofiznih adrenokortikotropa kod odraslih pacova Wistar soja. Dobijeni rezultati ukazuju na značajne promene u morfofunkcionalnim osobinama adrenokortikotropa nakon izlaganja toplotnom stresu, bez uočenih razlika u obliku i lokalizaciji ćelija u poređenju sa kontrolama. Volumen adrenokortikotropa kao i njihova volumenska gustina bili su značajno smanjeni ($p<0,05$) za 12,3% odnosno 26,7%, u poređenju sa kontrolom. Koncentracije adrenokortikotropnog hormona u plazmi i kortikosterona u serumu u eksperimentalnoj grupi bile su značajno smanjene za 21,9% odnosno 27,2%, u poređenju sa kontrolnim vrednostima. Navedeni rezultati pokazuju da 24-časovno izlaganje odraslih mužjaka pacova toplotnom stresu ima inhibirajući efekat na morfofunkcionalne osobine hipofiznih adrenokortikotropa.

Ključne reči: toplotni stres, ACTH, mužjaci pacova

Introduction

Global warming and its impact on average temperature elevation during the summer period in the southeastern parts of Europe, including Macedonia, represent an inevitable stress for all living organisms. Activation of the hypothalamic-pituitary-adrenal (HPA) system during exposure to various types of stressors is a well-known physiological concept. Heat stress was shown to be the strongest stressor, when compared to immobilization or cold stress (1, 2).

The critical moment of the ACTH response to acute stress implies a synergy between the most

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List of Abbreviations: ACTH, adrenocorticotrophic hormone; ACTH cells, pituitary adrenocorticotropes; AVP, arginine-vasopressin; CRH, corticotrophin-releasing hormone; HPA, hypothalamic-pituitary-adrenal; PAP, peroxidase-antiperoxidase complex; Vc, volume of cells; Vn, volume of nuclei; Vv, volume density.

stress-sensitive paraventriculo-infundibular corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) producing neurons (3, 4). ACTH, released into the peripheral circulation in response to stress, stimulates the synthesis and secretion of adrenal glucocorticoids (3, 5, 6). Studies carried out with rats demonstrate that the feedback effects between some hypothalamic peptides (CRH, AVP) and adrenocortical steroids influence the morphology, size, and number of pituitary ACTH cells (7, 8).

Plenty of data demonstrate that acute heat stress (35–41 °C) increases plasma levels of ACTH and serum corticosterone concentration after several minutes (9), or in the first 2–3 hours of exposure (10–13). On the other hand, data concerning the effects of a 24-hour exposure to heat stress are rather contradictory while reporting an increase (14, 15), decrease (11) or no changes (16, 17) in the ACTH and corticosterone blood concentrations. There is an assumption (hypothesis) of a three-phase reaction of the HPA axis upon thermal stress, consisting of the activation, suppression and normalization of the activity, somewhere below the normal value (18).

Taking all these facts into consideration, as well as the lack of data about the effect of high ambient temperature on the morphology of ACTH cells, the present study was focused on the morphological as well as hormone secreting characteristics of immunopositive ACTH cells in male rats, after one-day exposure to high ambient temperature (35±1 °C).

Material and Methods

Animals

The animals used in this study (both the control and the experimental group) were adult male Wistar rats (280–350 g), aged 3–4 months. The animals were kept under a 12:12 h light–dark regimen, with access to food and water *ad libitum* throughout the whole experiment.

The experimental group of rats was exposed to 35±1 °C for 24 h, in a specific thermal chamber, with controlled temperature and humidity of 30–40%, whereas the control group was kept at room temperature (20±2 °C). There were 7 animals per group.

The experimental protocols were approved by the Local Animal Care Committee in conformity with the recommendation provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

Light microscopy and immunocytochemistry

The pituitary glands were excised, fixed in Bouin's solution for 48 h and embedded in paraplast. Serial 5-μm thick tissue sections were deparaffinized in xylol and serial alcohol. Pituitary hormones were localized by the peroxidase-antiperoxidase complex (PAP) method of Sternberger et al. (19). The endogenous peroxidase activity was blocked by incubation in a 9 mmol hydrogen peroxide solution in methanol for 30 min at ambient temperature. Before the application of specific primary antisera, nonspecific background staining was achieved by incubation of the sections with nonimmune, i.e. normal porcine serum diluted with phosphate buffered saline (PBS) pH 7.4 for 60 min. Sections were then overlaid with appropriate dilutions of the specific primary antibodies (hACTH antisera, Dako A/S, Glostrup, Denmark) for 48 h at 4 °C. This antibody strongly cross-reacts with rat ACTH (Starčević et al. (20); verified by Dr B. A. Yang of Dako Corp). After washing in PBS for 5 min, sections were incubated for 60 min with a secondary antibody, swine anti-rabbit IgG (DAKO, Glostrup, Denmark; diluted 1:100 in PBS), rinsed again in PBS for 5 min and then incubated with rabbit PAP complex (DAKO A/S, Glostrup, Denmark; diluted 1:100 in PBS), for 45 min. Binding sites were visualised using 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany) and 0.03% hydrogen peroxide in 0.2 mol/L TRIS-HCl buffer, pH 7.4. The sections were counterstained with hematoxylin and mounted in Canada balsam (Molar Chemicals KFT, Budapest, Hungary). For the control sections, the primary antibody was omitted and replaced by PBS, pH 7.4.

Morphometry

Volume densities (Vv) of the nuclei and cytoplasm of ACTH-immunoreactive cells, as well as numerical density (Na) of their nuclei per μm^3 were measured using 50 test areas in the pituitary gland with a magnification of ×1000, using the multipurpose test system M_{42} (21).

The number of nuclei of immunoreactive ACTH-cells per mm^3 was estimated using Weibel and Gomez (21) formula according to Weibel (22). Since the rat ACTH cells are mononucleated, the numerical density of nuclei (Nv) corresponds to the number of cells per cubic millimeter.

$$Nv = (k/\beta) \times (Na^{3/2}/Vv^{1/2})$$

On the basis of earlier karyometric studies (23) the shape coefficient β was estimated to be 1.382, for the pituitary cells. It relates the Nv (number of cells counted per unit volume) to the Na (number of cells counted per square millimeter) and Vv (volume density) and depends on the axial ratio of the nuclei.

The volume densities of ACTH-positive cells were expressed as percentages of total pituitary cells in mm^3.

Hormonal analyses

Blood was collected from the trunk and separated plasma and sera samples from all animals were stored at the same time at –70 °C until assayed.

Plasma levels of ACTH were determined without diluting the plasma, by the IMMULITE method (DPC, Los Angeles, USA), in duplicate samples within a single assay, with an intra-assay CV of 9.6%. Serum corticosterone concentrations were determined without diluting the sera, by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate samples within a single assay, with an intra-assay CV of 8.0%.

Statistical analysis

The morphometric and biochemical data obtained for each group were averaged and the standard deviation of the mean was calculated by Student's t-test. A probability value of 5% or less was considered statistically significant.

Results and Discussion

Body weight, absolute and relative pituitary weights

Data for the body weight, the absolute and relative pituitary weights are summarized in *Table I*. The body weight in heat stressed rats was decreased ($p<0.05$) by 15.3%, compared to the controls. Our data are in agreement with the results from Mitev (18) and Katsumata and Yano (24) according to which the body weight is decreased regardless of the duration of heat exposure, demonstrated to be the consequence of food-intake reduction and increase of water consumption (25). After the heat stress the relative pituitary weight was significantly increased ($p<0.05$) by 16.5%, in comparison with the corresponding control (*Table I*). These findings are, primarily, the consequence of evidently decreased body mass.

Table I Body weight, absolute and relative pituitary weights in control and heat stressed rats.

Groups	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg%)
Control	337.5±26.9	6.5±0.58	2.5±0.1
Experimental group	285.8±13.2* (–15.3%)	6.8±0.6 (+4.6%)	2.9±0.2* (+16.0%)

The values are the means±S.D. for seven animals per group.*$p<0.05$ vs. control

Immunohistochemical and morphometric findings

The ACTH-immunopositive cells, in both groups, were mostly located in the central part of the pituitary *pars distalis*. In control males, they were present as small groups in close proximity to numerous capillaries. ACTH-immunopositivity was granular, uniformly distributed throughout the relatively small portion of cytoplasm surrounding the prominent nuclei. Corticotropes were ellipsoid or polygonal in shape, often with processes among neighboring cells (*Figure 1A*). In comparison to the controls, the ACTH-immunopositive cells in heat stressed rats were less numerous, darker and smaller, although their location and shape remained as in the controls (*Figure 1B*). Stereological analyses showed that the ACTH cells in control rats had a mean cell volume of 1091 μm^3, a nuclear volume of 189 μm^3 and they occupied 15.0% of the pituitary volume (*Figure 2A-C*). After exposure to heat stress, the volume of ACTH cells and their volume density were significantly decreased ($p<0.05$) by 12.3% and 26.7%, respectively, compared to the control group (*Figure 2A, C*).

Different studies have established a positive correlation between the morphological changes of the ACTH cells and their functional state. Investigations demonstrating an increased activity of ACTH cells, showed an increase in serum ACTH concentration, cellular area and immunopositive area of the

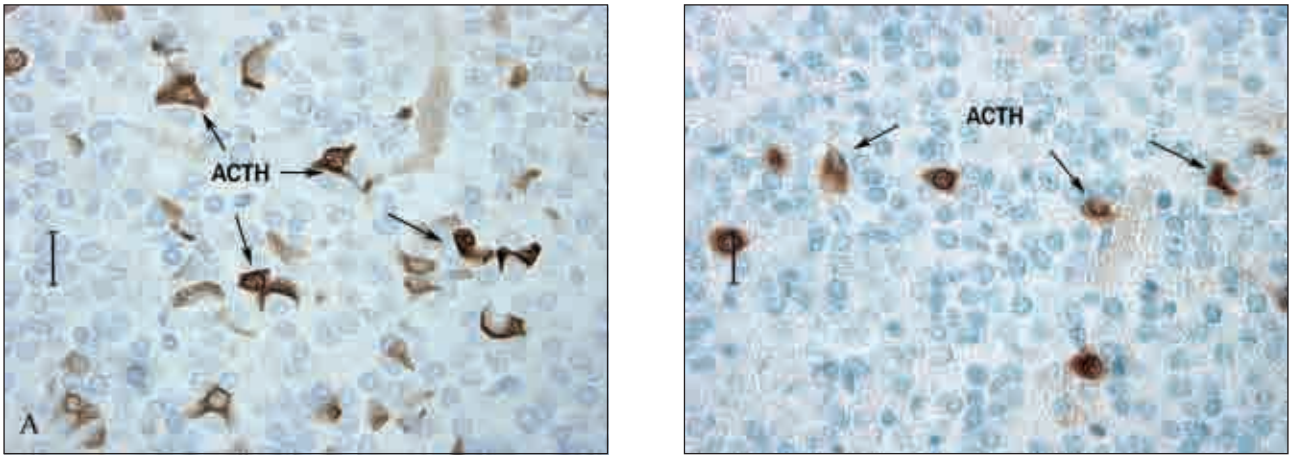

Figure 1 Immunopositive ACTH cells in: A) control rats; B) rats exposed to 35±1 °C. (PAP, bar 16 μm)

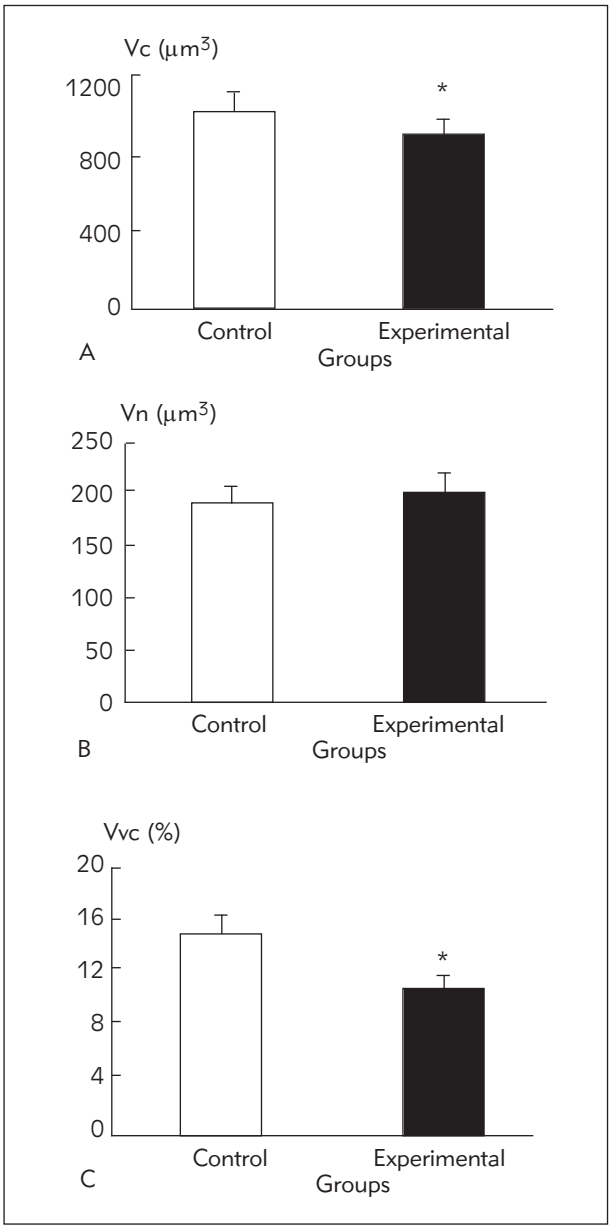

Figure 2 Morphometric parameters of ACTH cells in control and rats exposed to 35±1 °C: A) volume of cells (Vc; mm^3), B) volume of nuclei (Vn; μm^3), C) volume density (Vvc; %). The values are the means±SD (n =7), *p<0.05 vs. control.

pituitary ACTH cells, while decreased activity of these cells showed decreased values of those parameters in rats (7, 8) as well as in male viscachas (26). Our findings of decreased cellular volume as well as volume density of ACTH cells suggest a decreased activity of this type of cells, which may be, to some point, due to total or partial degranulation of ACTH cells after secreting its hormone. Fillipa and Mohamed (26) have suggested that preparation of the organism for different environmental conditions such as seasonal variations throughout the year, as a metabolic adjustment adequate for survival, underlies

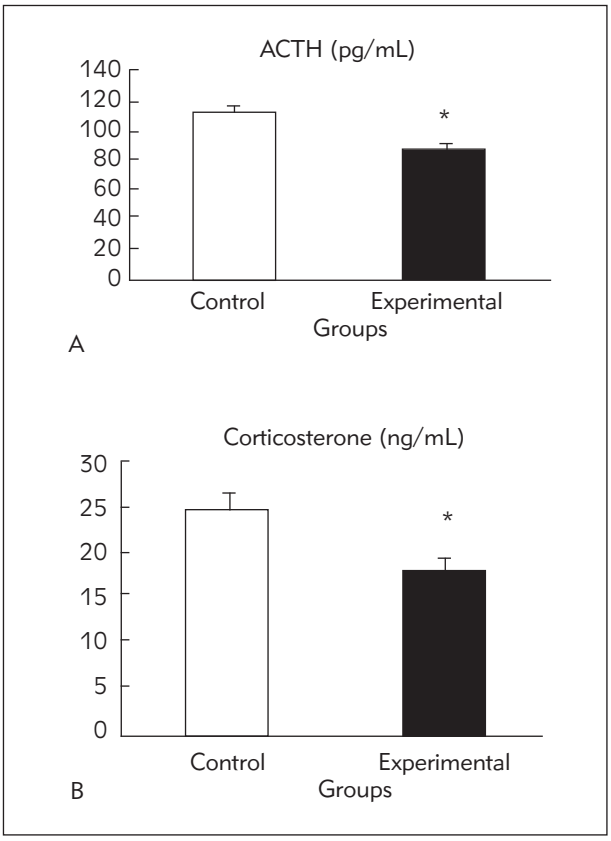

Figure 3 Hormonal concentrations in adult male rats. A. Plasma concentrations of ACTH (pg/mL). B. Serum concentrations of corticosterone (pg/mL). Data are expressed as the means±SD (n =7), *p<0.05 vs. control.

these findings. According to them, when ACTH cells start to recover, these degranulated cells will start to store secretory granules again.

Plasma ACTH and serum corticosterone level

The ACTH and corticosterone concentrations in the blood of heat stressed rats obtained in our study were found to be significantly reduced (p<0.05), by 21.9% and 27.2%, respectively, in comparison with the corresponding values in the control group (*Figure 3*). Decreased plasma ACTH concentration, till the control values, was reported during the 24-hour heat exposure study (16). Considering the data about decreased concentration of serum corticosterone found in this study, we assume that the decreased activity of ACTH cells may be due, in some part, to the negative feed-back mechanism of glucocorticoids upon the HPA axis, and therefore, upon ACTH cells. Generally, there is a synergistic relationship between the adrenal and thyroid gland through the HP axis (27, 28). The data from Rousset et al. (25) have shown suppression of thyroid activity in the first two days of exposure to 34 °C, and therefore decreased activity of the CRH and ACTH (27). Our findings of a decreased plasma concentration of ACTH, as well as the decreased cell

volume and volume density of the ACTH cells, might be, in some degree, the result of decreased activity of CRH via the thyroid gland. Decreased corticosterone level in our study is in accordance with the findings of Bertin (29), who have reported that the exposure to moderate heat caused a decrease in adrenocortical activity as evaluated by the fall of both adrenal and plasma corticosterone levels. Low corticosterone levels in rats exposed to 35 °C for 24 hours were also reported, indicating depressed adrenocortical activity (30, 11, 31). Furthermore, the latter authors have shown that the transient increase in corticosterone serum concentration, 1–3 hours after heat exposure, is followed by a decrease below the control value in the next 24 hours. According to these authors, the first reaction to heat has some similarity to the pattern of the »alarm« reaction, but 24 hours of exposure to a hot environment produced suppression in adrenocortical activity.

The findings revealed in this study suggest that one day exposure of adult male rats to high ambient temperature (35±1 °C) has an inhibitory effect on the morphological and hormone secreting characteristics of ACTH cells in adult male rats.

Acknowledgements. Thanks are due to Olga Stefanović (Vicor D.O.O., New Belgrade, Serbia) and to Dragan Ristić (Laviefarm D.O.O., Belgrade, Serbia) for technical support. This work was supported by the Ministry of Education and Science, Republic of Serbia, Grant number 193007.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: March 22, 2011

Accepted: April 19, 2011