Altered Hypothalamic-Pituitary-Adrenal Axis Activity in Patients with Chronic Heart Failure

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Key words
- HPA axis
- corticotrophin-releasing hormone
- ACTH
- cortisol
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Abstract

Neuroendocrine factors play an important role in the pathogenesis of chronic heart failure. Despite numerous clinical and experimental studies, the role of the hypothalamic-pituitary-adrenal axis and glucocorticoid hormones is not fully characterised. Here we present a study of plasma cortisol concentration in 74 chronic heart failure patients, divided into four groups based on NYHA functional classes I–IV, and in 17 control subjects. In parallel, we performed morphological analysis of the hypothalamic-pituitary-adrenal axis components from 8 male patients who had died from chronic heart failure, and 9 male controls. In our study we applied immunohistochemical method and quantitative analysis to investigate an expression of hypothalamic neurohormones (corticotropin-releasing hormone, vasopressin) and adrenocorticotropin hormone in the pituitary, as well as performed general histological examination of the adrenal cortex. Measurement of morning cortisol concentration in plasma of chronic heart failure patients revealed neither difference compared to controls nor with the severity of the disease. Despite this, a two-fold increase in the density of corticotropin-releasing hormone-immunoreactive neurons as well as a two-fold increase in the number of corticotropin-releasing hormone neurons co-expressing vasopressin in the hypothalamic paraventricular nucleus were found. In the anterior pituitary the density of adrenocorticotropin hormone-immunoreactive cells was significantly increased. General histological analysis of the adrenal cortex revealed a drastic thinning of the zona fasciculata and dystrophic changes in corticocytes. Structural changes, observed in the adrenal cortex, suggest a relative glucocorticoid deficiency, which may contribute to corticotropin-releasing hormone and adrenocorticotropin hormone upregulation in hypothalamus and pituitary of chronic heart failure patients.

Introduction

Chronic heart failure (CHF) is a widely distributed disorder, expected to become even more prevalent in the near future because of the aging of the population [1]. According to the neurohormonal hypothesis [2], CHF is considered now as a highly complex clinical syndrome, and its manifestation and progress are strongly dependent on the activation of hormones and cytokines ([3] and references cited therein). Various neuroendocrine factors, such as norepinephrine, angiotensin II, rennin, and endothelin are strongly activated in CHF, and along with mechanical signals they cause an increase in myocyte size and hypertrophy of the heart ([4] and references cited therein). Vasopressin (VP) was also considered as an important neuroendocrine pathogenetic factor of CHF. VP is synthesized mainly in magnocellular hypothalamic nuclei and released into peripheral blood circuitry from the posterior pituitary lobe. However, VP is also produced by parvocellular neurons in the paraventricular nucleus (PVN) (for review, see [5]), where VP co-localizes with corticotropin-releasing hormone (CRH) and potentiates effects of CRH on pituitary corticotrophs, especially under chronic stress situation [6, 7].

Despite the fact that the role of several neuroendocrine systems has been extensively studied in CHF patients, the contribution of the hypothalamic-pituitary-adrenal (HPA) axis to the pathogenesis of CHF is not fully understood. The HPA axis is the key biological machinery, which is primarily activated in response to stress and regulated in a hierarchical manner via positive and negative feedback mechanisms [8]. With respect

to CHF, the literature on the activity of the HPA axis is controversial: measurement of cortisol in the plasma of CHF patients revealed either normal concentrations [9, 10], decreased [11, 12], or increased levels [13, 14]. However, recent comprehensive study [15] performed on a total of 300 patients afflicted by CHF of New York Heart Association functional classes III or IV or systolic heart failure showed that all these patients have a normal range of plasma cortisol levels comparable to those in healthy individuals [16]. Nevertheless, higher plasma cortisol (within in a normal range of cortisol concentrations) had a positive correlation with higher mortality risk in CHF patients [15]. The pituitary function of CHF patients has been examined by only one group [17], which showed elevated adrenocorticotropin hormone (ACTH) levels in the plasma. While the responses of pituitary to CRH as well as the basal activity of CRH neurons in the hypothalamic PVN have not been studied in CHF patients, evidence from animal models of CHF strongly suggested an upregulation of CRH expression [18, 19].

The aim of the present work was the analysis of plasma cortisol concentrations in CHF patients combined with postmortem morphological examinations of hypothalami, pituitary, and adrenal glands. In the course of this study we found no changes in cortisol plasma concentration in CHF patients, even with severe form [III, IV functional classes, according to the New York Heart Association (NYHA) criteria], which was accompanied by dystrophic changes in the adrenal cortex. These findings coincided with a profound increase of CRH expression in the hypothalamus and ACTH expression in the pituitary, suggesting a disbalance in feed-back signalling in the HPA axis of CHF patients.

Materials and Methods

Measurement of plasma cortisol concentrations

Seventy-four patients of both genders (41 males and 33 females), ranging in age from 32–88 years (64.8 ± 11.5 years) were divided into groups based on functional classes (I: 4 patients; II: 19 patients; III: 23 patients; IV: 28 patients) of CHF according to the NYHA criteria. Patients with myocardial infarction within the previous 12 months were excluded. The control group contained blood samples from 17 individuals (8 males and 9 females; age ranging from 45 to 68 years; mean age 60.8 ± 4.98 years) without known neurological, psychiatric or endocrinological diseases. These patients had initial signs of CHF, such as slight dyspnoea during physical activity, but did not have other symptoms to assess any stage of CHF. Blood samples from all patients were collected at the same time in the morning (8–10 AM). Plasma cortisol levels were measured by enzyme immunoassays (DRG® Cortisol ELISA Kit, DRG International, Inc., Mountainside, NJ, USA). Written informed consent was obtained from all patients before the study. The study was in agreement with the guidelines approved by the ethics committee of the Russian State Medical University (Moscow, Russia).

Postmortem morphological study

Tissue collection

Brains from patients were obtained according to the Russian law at autopsy from the Regional Medical Expert Bureau of Medical Legal Examination (Kaliningrad, Russia) and from an Emergency Hospital (Kursk, Russia). Some samples of adrenal glands were obtained from Department of Pathology (Bureau of Pathological Anatomy), Regional Hospital (Belgorod, Russia). The study was approved by the Local Ethics Committee of Friedrich-Schiller University, Jena, Germany (Protocol 2387-09/08). Brains from eight male patients who had CHF, ranging in age from 35–65 years (53.8 ± 9.3 years), were obtained at autopsy. In all cases CHF was verified clinically and/or by autopsy by several pathological criteria: hypertrophy and lipomatosis of the myocardium with dilated heart chambers; local hypertrophy, dystrophy, or atrophy of cardiomyocytes with their lysis and subsequent cardiosclerosis; injuries of blood capillaries with an increase of the wall thickness; and activation of fibroblasts. Patients had died from acute heart or respiratory failure (acute cardiovascular insufficiency). The control group contained samples of brains from nine male individuals, ranging in age from 36–70 years (54.6 ± 11.8 years), without known primary neurological or psychiatric conditions and endocrinological pathology who died from non-CHF related causes: acute brain stroke (3), diseases of the respiratory system (2), acute surgical pathology (2), asphyxia (1), and traumatic injury (1). Postmortem time was 15.8 ± 6.4 h in controls and 22.5 ± 11.7 h in CHF group. Tissue preparation was performed as described [20]. Briefly, hypothalami, pituitary and adrenal glands were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at room temperature for about 2 months. Tissue samples were dehydrated in graded ethanol, embedded in paraffin, cut into serial 6–8 µm coronal or horizontal sections and collected on a series of 10 SuperFrost® Plus glass slides (Roth, Karlsruhe, Germany).

Immunohistochemistry

Consecutive sections of pituitaries and hypothalami containing PVN were stained with antibodies against ACTH or CRH as described [20]. Briefly, after deparaffinisation and rehydration, sections were incubated in phosphate-buffered saline (PBS) containing 10% methanol and 3% hydrogen peroxide. After preincubation with 0.05% Triton X 100-PBS containing 5% normal goat serum (PBS-NGS), sections were incubated overnight with one of the primary rabbit polyclonal antibodies – anti-CRH (1:1 000, Peninsula Labs Inc.) or anti-ACTH (1:200, Chemicon International, CA, USA) in working solution (WS; 1% NGS, 0.05% Triton on PBS, pH 7.4). Then sections were washed with PBS and incubated in WS with biotinylated goat anti-rabbit IgG (1:200, Vector Elite kit, Vector Laboratories, Inc., Burlingame, CA, USA). After washing with PBS, ABC complex (Vector Laboratories, CA, USA) in PBS was applied. The reaction was visualised with diaminobenzidine (DAB, Sigma) and H_2O_2 in PBS. After rinsing with distilled water, sections were dehydrated in ethanol, cleared in xylene and mounted with embedding medium (Entellan, Merck, Rahway, NJ, USA). For control incubations we used NGS, diluted to 1:1 000 in PBS-Triton X instead of the primary antibodies. Sections of adrenal glands were stained with hematoxylin-eosin as described [20]. To evaluate co-localization of CRH with VP double fluorescent immunostainings were performed. Slides were preincubated with 1% Triton X 100-PBS containing 10% NGS and then with the mixture of the primary antibodies: rabbit polyclonal anti-CRH; Peninsula Labs Inc., 1:200 and mouse monoclonal PS41 anti-VP, 1:100, provided by Dr. Harold Gainer [21, 22], dissolved in 0.5% Triton X 100-PBS containing 1% NGS (WS). Immunosignals for CRH and VP were visualised by a Cy3-conjugated goat anti-rabbit and a FITC-conjugated goat anti-mouse IgG (1:100 in WS, Dianova,). Sections were mounted with antifading

medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, Germany).

Semiquantitative analysis

Counting of CRH-positive neurons was performed on an Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with a motorised stage and Neurolucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany). The contours of the PVN field occupied by CRH-positive neurons were outlined manually with the cursor using a 5× objective (Plan-Neofluar®). All immunopositive neurons containing a nucleus and clearly distinguishable from background within the selected area were counted using a 20× objective [23]. Cells were labeled by a symbol and their numbers as well as an outlined area were estimated automatically. Finally, cell profile densities (numbers of cell profiles per unit area) of CRH-positive cells in each subject were calculated based on the total number of counted neurons in the sample and the estimated sample area [24]. Additionally, average nearest neighbour distances between immunolabeled neurons were calculated using Neuroexplorer software (MicroBrightField Europe, Magdeburg, Germany). At least three hypothalamic sections from each subject were analysed.

Counting of double-labeled CRH/VP neurons was performed on the digital images of the immunostained sections obtained with a confocal laser scanning microscope TCS SP5 (Leica, 20× objective, 1024×1024 pixels resolution) using the "Image Tool 2.0" software. Stacks of images of 1 µm thickness were obtained from different parts of the PVN in sections double-stained for CRH and VP. Then, the three-dimensional reconstruction of the Z-series was performed to assess an overlap between CRH and VP for confirmation of co-localized immunoproducts. The total number of CRH-positive neurons and the number of CRH neurons immunopositive for VP were counted. Then the percentage of single and double-labeled CRH-positive cells was calculated. All counts were blindly performed on coded preparations by the same investigator.

Counting of ACTH-labeled cells in the anterior lobe of pituitary was performed on the digital images of the immunostained sections obtained with a digital camera "Olympus DP10" with the "DP-Soft 3.0" software (20× objective) using the "Image Tool 2.0" software (University of Texas Health Science Center, San Antonio, Texas). The number of all immunopositive cells within the defined area (100 µm × 100 µm) was estimated and then the cell profile density was calculated. From each subject nine fields were analysed in three sections.

Photographical documentation

Immunohistochemically stained sections with DAB visualisation of final products were examined with an "Olympus BX50" microscope. An "Olympus DP10" digital camera with "DP Soft 3.0" software was used for microphotography. Digital images were adjusted for contrast and brightness in "Adobe Photoshop" (Adobe Systems, version 8.0.1).

Statistical analysis

The differences in blood cortisol concentrations were analysed by one-way analysis of variance (ANOVA) for repeated measures using the online program http://faculty.vassar.edu. Two-tailed parametric test (*t*-test) was applied to evaluate the morphological data using "MS Excel-7.0" tool. All results are presented as a group mean values with standard deviation (SD). The accepted level of significance was 5%.

Results

Plasma cortisol concentrations

Measurement of total plasma cortisol revealed no significant sex differences either in controls (138.38 ± 36.65 ng/ml, males and 148.62 ± 41.79 ng/ml, females) or CHF patients (130.09 ± 59.52 ng/ml and 132.16 ± 49.38 ng/ml, in males and females, respectively; values in patients of different functional classes of CHF were pooled). Comparison of cortisol levels between controls and CHF patients revealed no significant difference (143.8 ± 41.01 ng/ml, controls and 131.01 ± 55.24 ng/ml, CHF patients). There were no significant changes in cortisol levels between the groups of CHF patients divided into four functional classes of CHF according to the NYHA classification (121.28 ± 21.96 ng/ml, 121.55 ± 46.05 ng/ml, 122.43 ± 38.2 ng/ml and 145.88 ± 70.72 ng/ml, in patients with I, II, III, and IV functional class of CHF, respectively).

CRH neurons

In the PVN of control patients small and medium sized CRH-positive neurons were located along the third ventricle (○ **Fig. 1A**). The intensity of CRH-staining varied from poor to very strong, but weakly and moderately stained cells prevailed (○ **Fig. 1C**). In CHF patients, the population of CRH-positive neurons in the PVN was much larger in comparison with the control group (○ **Fig. 1B**). CRH-immunoreactivity (IR) appeared as clear homogeneous cytoplasmic staining pattern. Most CRH-positive perikarya as well as their processes were intensively stained (○ **Fig. 1D**). Results of the quantitative analysis indicate that the profile density of CRH-positive neurons in the PVN is significantly increased in CHF patients (+70%; $p < 0.05$, *t*-test), as shown in ○ **Fig. 1E**. A nearest neighbour distance analysis was also performed to obtain insight into the spatial distribution of CRH-labeled neurons. The average nearest neighbour distance between CRH-positive cell profiles in PVN was significantly smaller in CHF patients (–21%, $p < 0.05$, *t*-test, ○ **Fig. 1F**). This finding correlates well with the estimates of cell profile density (○ **Fig. 1E**). In order to evaluate co-expression of CRH and VP in the same neurons we performed double immunofluorescent staining (○ **Fig. 2**). As indicated in the graph (○ **Fig. 2C**), CRH-IR was found in 24.9 ± 2.6% of the VP-positive neurons in controls (○ **Fig. 2A**). In CHF patients, the proportion of double-labeled neurons was increased almost two-fold as compared with the controls (○ **Fig. 2B, C**).

Anterior pituitary

In cells of the anterior lobe of pituitary ACTH-IR was found as a homogenous cytoplasmic staining pattern. The intensity of the IR signal was much more pronounced in patients with CHF as compared with the controls (data not shown). Semiquantitative analysis revealed a significant increase (+41%, $p < 0.001$) in relative density of ACTH-positive cells in the anterior lobe of patients afflicted with CHF.

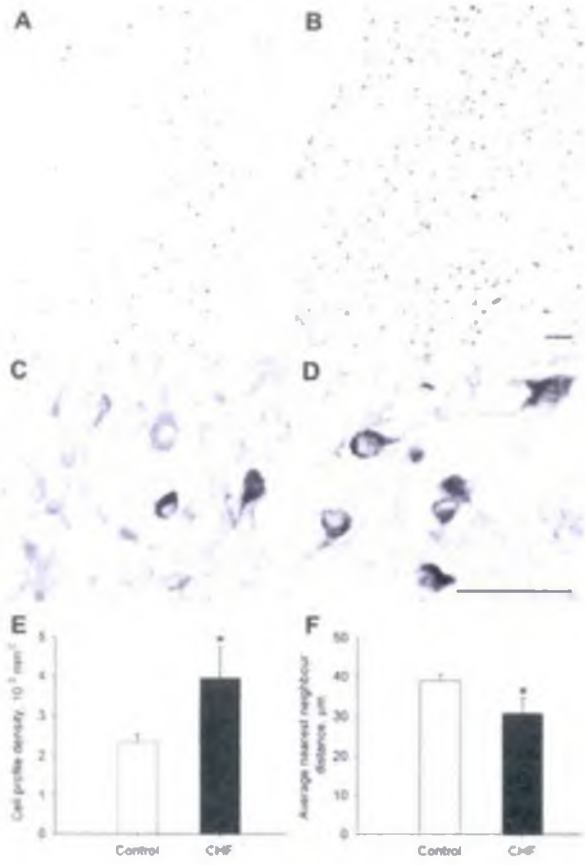

Fig. 1 Increased CRH-immunoreactivity in parvocellular neurons of the PVN in CHF patients. **A, C**: CRH-positive neurons in a 64-year old control patient, at small (**A**) and big (**C**) magnifications. **B, D**: Numerous intensively stained CRH-positive neurons in the PVN of a 65-year old patient afflicted with the CHF, at small (**B**) and big (**D**) magnifications. Immunohistochemical staining with anti-CRH antibodies, developed by DAB. Scale bars = 100 µm (**A, B**) and 50 µm (**C, D**). **E**: Relative profile density of CRH-positive cells is significantly higher in CHF group than in controls. **F**: The average nearest neighbour distance between CRH-positive cell profiles in PVN is significantly smaller in CHF patients. Group mean values of the parameters are calculated from individual mean values. * $p < 0.05$ (*t*-test).

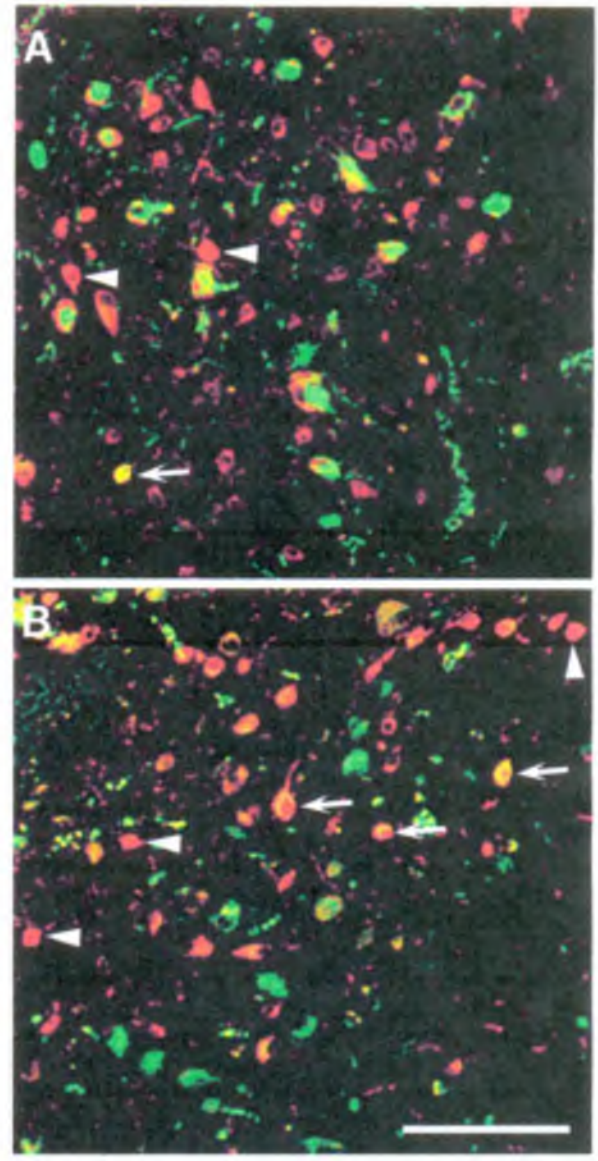

Fig. 2 Increased VP-immunoreactivity in CRH neurons of CHF patients. A: Fragment of the hypothalamic PVN of the 64-year old control and 62-year old CHF patients (**B**). Immunohistochemical double staining with rabbit anti-CRH and mouse anti-VP antibodies visualised with fluorescent Cy3- or FITC-conjugated secondary antibodies, respectively. Arrows indicate parvocellular CRH neurons containing VP-immunoreactivity. Single-labeled CRH-positive cells are indicated by arrowheads. Scale bar = 100 µm. The images are maximal projection Z-stacks of seven (in **A**) and eight (in **B**) confocal sections separated by 1 µm.

Adrenal cortex

In CHF patients we observed drastic thinning, plethora and diapedesis of the zona fasciculata as well as a discomplexation of the zona glomerulosa (○ **Fig. 3B**), as compared with controls (○ **Fig. 3A**). Detailed examination of the zona fasciculata revealed a profound vacuolated dystrophy (○ **Fig. 3C**), pyknosis (○ **Fig. 3C, D**) and delipidation of corticocytes in the presence of erythrostasis (○ **Fig. 3D**).

Discussion

The measurement of CRH-IR in the postmortem hypothalami has been performed previously by several groups, which showed reliable changes of hypothalamic CRH expression in several human diseases such as depression [25], multiple sclerosis [26], hypertension [23, 27], and alcoholic disease [20]. Moreover, ACTH-IR has been also immunohistochemically studied in the postmortem hypothalamus and hippocampal formation in temporal lobe epilepsy patients [28]. In the present study, comprising by the cases with relatively similar postmortem period in control and CHF group, we observed a profound activation of CRH and ACTH expression in human patients afflicted with CHF. These facts are in line with the results from animal models of CHF, showing that in rats with ischemia-induced CHF the metabolic [29] and neuronal [30] PVN activity was increased. More specifically, CRH expression in the PVN was significantly elevated

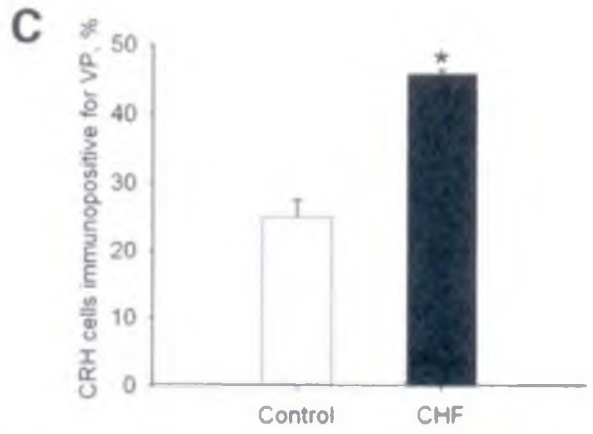

Fig. 2 (Continued) **C**: Proportion of CRH-positive neurons immunopositive for VP is strongly increased in CHF patients. Group mean values of the parameters are calculated from individual mean values. *$p<0.05$ (*t*-test).

in CHF rats compared with sham-operated animals and CRH protein expression in hypothalamus was 1.9-fold higher in CHF than sham rats as indicated by immunohistochemistry and Western blot analysis [18]. The increase in number of CRH neurons in the PVN was well correlated with the elevation of plasma ACTH [19]. All these observations are in agreement with our results in human patients with demonstrated elevation of CRH expression and increased numbers of CRH cells co-expressing VP, which potentiates effects of CRH on ACTH release [31]. Furthermore, results of the present study revealed not only a significant increase in the density of CRH neurons, but also an enhanced intensity of the staining in the PVN of CHF patients. These observations can serve as evidence for a drastic increase in CRH synthesis: according to recent reports a two-fold increase in CRH-IR cell number in the PVN of hypertensive patients corresponded to a five-fold elevation of CRH mRNA levels [23,27]. However, immunohistochemical technique applied to our study does not allow us to definitively conclude that basal CRH release into the portal blood is increased in CHF patients. Therefore, further measurements of CRH mRNA levels in the hypothalamus and CRH receptor type 1 mRNA levels in the pituitary of CHF patients will be required.

The upregulation of CRH and VP expression in CRH neurons and ACTH expression in the pituitary observed in our study predicted the elevation of cortisol in the blood. However, the level of cortisol in our CHF patients did not differ from the control group, and no rise of cortisol was found in patients with severe forms of CHF. These results are consistent with previous clinical data showing similar plasma cortisol concentrations in CHF patients and healthy subjects [9] and the absence of correlation of cortisol levels with the hemodynamic parameters [10]. In contrast, several groups reported a significant elevation of cortisol in CHF patients compared to controls and a progressive increase of its concentrations commensurate with the functional class of CHF [13,14]. However, profound dystrophic changes in corticocytes of the zona fasculata, detected in our histological study of adrenal cortex, support recent convincing data demonstrating either a normal range of cortisol concentrations in CHF patients [15] or even decreased levels of cortisol in some of CHF patients [12]. Furthermore our clinical and morphological results are in the line with the reported decline of cortisol responses to physical

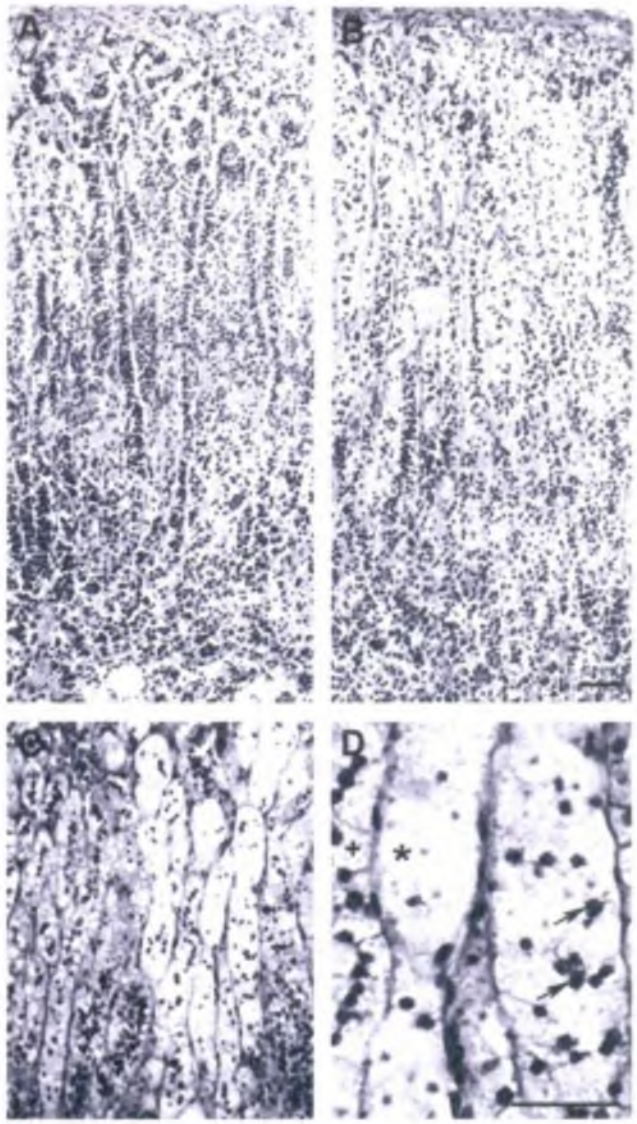

Fig. 3 Pathomorphological changes in the adrenal cortex. **A**: The structure of the adrenal cortex of the 65-year old control and 62-year old CHF patients (**B–D**). **B**: discomplexation of zona glomerulosa; drastic thinning, plethora and diapedesis in zona fasciculate (indicated by asterisk). **C**: Profound vacuolated dystrophy, pyknotic changes in corticocytes of zona fasciculata. **D**: Erythrostasis, delipidation (big asterisk; "normal" neighbouring tissue indicated by plus), pyknotic changes in corticocytes (arrows) of zona fasciculata. Staining with hematoxylin-eosin. Scale bars = 100 μm (**A**, **B**, **C**), 50 μm (**D**).

exercises in CHF patients [11]. The lack of morphological evidence for adrenal cortex hyperstimulation in CHF patients, as well as a normal basal cortisol levels coincided with increased ACTH-content in the pituitary may suggest several possibilities, such as the block of ACTH release from pituitary, decrease of sensitivity of adrenal cortex to ACTH, or interception of ACTH action with other modulators. To dissect these possible mechanisms, further studies of ACTH receptor expression in the adrenals as well as low-dose ACTH stimulation test in CHF patients will be very informative.

The discrepancy between the CRH production and plasma glucocorticoid levels has been observed in several human pathologies and some experimental models. For example, the elevation of CRH production without reported increase in plasma cortisol

levels has been shown in alcoholics [20, 32] and patients with multiple sclerosis [26, 33]. In opposite, the elevated cortisol levels coincided with the unchanged hypothalamic CRH expression in patients with Alzheimer disease [34, 35]. In animal models the discrepancy between CRH, ACTH, and corticosterone responses has been earlier observed in rats with chronic inflammation [36]. In these rats subjected to repeated injections of bacterial endotoxin lypopolysaccharide a prominent elevation of basal CRH expression and a trend for elevation of ACTH expression coincided with unchanged ACTH and corticosterone plasma levels [6]. The dysregulation between the central and peripheral components of the HPA axis is a very important question, which will be addressed in our further studies with employment of in situ hybridisation technique for evaluation of CRH and ACTH receptors mRNA levels and performance of functional tests, such as very low-doses ACTH stimulation [36] and dexamethasone suppression tests [37] with subsequent comparison of total and free fractions of plasma cortisol [38] in CHF patients.

In conclusion, our study demonstrates that CHF patients exhibit the activation of hypothalamic and pituitary levels of the HPA axis but a failure of adrenal response and subsequent increase of systemic cortisol levels is noted. The unchanged plasma cortisol as well as severe morphological impairments of the adrenal cortex suggests that a primary adrenal locus is a principal site of the HPA axis dysregulation in CHF patients. The alteration of the adrenal cortex may lead to a mild (relative) deficiency of glucocorticoid production, which via feed-back signalling may lead to the upregulation of hypothalamic CRH expression in CHF patients.

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