ARTICLE IN PRESS

Acta Histochemica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Histological and morphofunctional parameters of the hypothalamic–pituitary–adrenal system are sensitive to daidzein treatment in the adult rat

Svetlana Trifunović[a,*], Milica Manojlović-Stojanoski[a], Nataša Nestorović[a], Nataša Ristić[a], Branka Šošić-Jurjević[a], Lazo Pendovski[b], Verica Milošević[a]

[a] University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Cytology, Belgrade, Serbia
[b] Cyril and Methodius University in Skopje, Faculty of Veterinary Medicine, Skopje, FYR, Macedonia

A R T I C L E I N F O

Keywords:
daidzein
HPA system
histology
stereology
biochemistry

A B S T R A C T

The isoflavone, daidzein is a biologically active, plant-derived compound that interacts with estrogen receptors. Data from previous studies have suggested that daidzein exerts beneficial effects in many diseases; however, as an endocrine disrupter, it may also alter the functioning of the endocrine system. Data regarding the effect of daidzein on the morphofunctional and histological parameters of the hypothalamic–pituitary–adrenal (HPA) system is still lacking. Therefore, using the newCAST stereological software, we investigated the effects of chronic (21 days) daidzein treatment on corticotropin-releasing hormone (CRH) neurons within the hypothalamus and corticotropes (ACTH cells) in the pituitary, while image analysis was employed to-examine the intensity of fluorescence of CRH in the median eminence (ME) and adrenocorticotropin hormone in the pituitary in adult orchidectomized (Ovx) rats. Circulating ACTH and corticosterone levels were also analyzed. This study showed that daidzein treatment decreased the volume density of CRH neurons within the paraventricular nucleus as well as CRH immunofluorescence in the ME. The total number of ACTH cells was decreased, while ACTH cell volume and the intensity of ACTH fluorescence were increased following daidzein treatment. Both ACTH and corticosterone blood levels were increased after daidzein administration. The results of performed experiments clearly demonstrate that volume density of CRH neurons; total number and volume of ACTH cells, as well as stress hormones levels are vulnerable to the effects of daidzein.

1. Introduction

Activation of the hypothalamic–pituitary–adrenal (HPA) system is considered to be a characteristic response to physical or psychological stress, the purpose of which is to maintain equilibrium between organisms and their environment (de Kloet et al., 2005). Stress-related inputs are integrated in the paraventricular nucleus (PVN) of the hypothalamus to induce the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which stimulate adrenocorticotropic hormone (ACTH) secretion from the pituitary, subsequently inducing glucocorticoids production by the adrenals. The HPA system activation is terminated by a negative feedback action of glucocorticoids: elevated levels of glucocorticoids decrease the synthesis of AVP, CRH and ACTH in a direct and indirect manner through the pituitary, hypothalamus and hippocampus (Whitnall, 1993). The activity of the HPA system shows pronounced sex-related differences (glucocorticoid levels are thus higher in females than in males) and estradiol is believed to play a causal modulatory role (Handa et al., 1994). Inter alia, estradiol may directly enhance CRH gene transcription in the hypothalamus via binding to estrogen-responsive elements on the CRH gene (Vamvakopoulos and Chrousos, 1993).

Phytoestrogens are estrogens that present in some plant. They are structurally and functionally similar to estrogens (Patisaul and Jefferson, 2010). Daidzein, one of the major isoflavones in soybeans, works by binding to estrogen receptors (ER) (Kuiper et al., 1998). Previous studies have revealed that daidzein exerts beneficial effects such as: improvement of preovulatory follicles development (Liu and Zhang, 2008), anti-cancer (Messina et al., 1994) and neuroprotective effects (Zhang et al., 2002), has antioxidative properties (Dwiecki et al., 2009), and that it effects could be dose-dependant (Benassayag et al., 2002). On the contrary, in view of estrogen's essential role in growth, differentiation and homeostasis, isoflavones may be classified as endocrine disruptors since they alter the normal functioning of the endocrine system (Henley and Korach, 2010).

* Corresponding author at: Department of Cytology, Institute for Biological Research "Siniša Stanković", 142, despot Stefan, Blvd., 11060 Belgrade, Serbia.
E-mail address: lanat@ibiss.bg.ac.rs (S. Trifunović).

https://doi.org/10.1016/j.acthis.2017.12.006
Received 12 October 2017; Received in revised form 12 December 2017; Accepted 12 December 2017
0065-1281/

Please cite this article as: Trifunović, S., Acta Histochemica (2017), https://doi.org/10.1016/j.acthis.2017.12.006

And yet, few experiments have been conducted to assess the daidzein effects on the HPA system and, to our knowledge, none have examined the effects of daidzein on stereological parameters at the hypothalamic–PVN and pituitary–ACTH levels by using the newCAST stereological system in adult rats. This study was undertaken to provide additional insight into the actions of daidzein by analyzing its effects on the volume of PVN, volume density of CRH neurons, and the volume density, volume and total number of ACTH cells in adult rats, considering the importance of the stereology approach as essential in the discovery of new concepts in cell biology. In addition, the blood concentrations of ACTH and corticosterone were also determined.

2. Material and methods

All animal procedures complied with the EEC Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research, 'Sinisa Stankovi' University of Belgrade, Serbia.

The experiments were performed on adult, 2-month-old male Wistar rats, kept in the facilities of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Under standard environmental conditions (a 12 h light\dark cycle, 22 ± 2° C), the animals were kept in groups of two per cage. Before entering the experiment, all the animals were fed the standard chow diet for laboratory rats (Veterinary Institute Subotica, Serbia). Two weeks prior to the start of the experiment the rats were fed a soy-free diet prepared in cooperation with the Department of Food, School of Veterinary Medicine, Belgrade, Serbia, and INSHRA PKB, Belgrade, Serbia, according to Picherit et al. (2000). Food and water were available ad libitum.

Under anesthesia (ketamine hydrochloride 15 mg/kg b.w.), the animals were orchidectomized (Orx) or sham-operated (So). Given that sex steroids act on the whole HPA system, by orchidectomy we want to minimize steroids influence and separate the effects of sex steroid and daidzein. The first group of Orx rats was subcutaneously injected with daidzein (D) (Nutraceutica) (30 mg/kg b.w.) every day at 9 AM, for 3 weeks, two weeks after surgery. The applied dose of daidzein (30 mg/kg b.w.) was chosen to mimic human exposure to elevated concentrations of isoflavones when nutritional supplements are used for therapeutic purposes (Doerge and Sheehan, 2002). Daidzein was dissolved in a minimal volume of absolute ethanol (0.1 ml) and then mixed with sterile olive oil (0.2 ml). The So and the second Orx group received the same volume of absolute ethanol in sterile olive oil and served as controls. The injection strategy is equivalent to oral consummation in terms of absorption rate (Jefferson et al., 2007) and provides an easy control of the applied dose. All rats were euthanized by decapitation under low ether anesthesia (ether ad narcosis Ph. Iug. III., Lek, Ljubljana, Slovenia) 24 h after the last injection. The low ether anesthetic provides relief from pain and tension (we used this anesthetic for 2 min), and doesn't affect ACTH and corticosterone hormone levels (de Haan et al., 2002).

2.1. Tissue preparation

The hypothalamus and pituitary glands were excised, fixed in Bouin's solution and dehydrated in increasing concentrations of ethanol and xylene. After embedding in Histowax (Histolab Product AB, Göteborg, Sweden), coronal serial sections of the hypothalamus (5 μm thick) and a serial section of the pituitary (3 μm thick) were obtained with a rotary microtome (RM 2125RT Leica, Glostrup, Denmark). Sakura Tissue-Tek Accu-Edge Low-Profile microtome blades for extremely thin sectioning were used. We used cresyl violet acetate solution to identify nuclear structures within the hypothalamus.

2.2. Immunohistochemistry

Hypothalamic CRH-containing neurons in the PVN and CRH immunoreactivity in the median eminence (ME) were determined using immunofluorescence. After dewaxing, hydration and rinsing in 0.01 M phosphate-buffered saline (PBS; pH 7.6 for 10 min), the sections were exposed to microwaves (700 W) in 0.05 M citrate-buffered (pH 6.0; for 2 × 10 min) for antigen retrieval. Subsequently, the sections were washed in PBS (3 × 10 min). To block nonspecific staining, the section were preincubated in normal donkey serum (1:10) for 30 min, and then incubated overnight with rabbit anti-CRH (1:500 in PBS; ab8901-100 Abcam). After washing in PBS, the sections were incubated with donkey anti-rabbit Alexa fluor 488 IgG (1:200; Invitrogen) for 2 h, then washed in PBS and mounted in mowiol. Antibody specificity has been evaluated by using blocking peptides (Trifunović et al., 2012) and the negative control and the results ware complete loss of immunoreactivity within PVN. Precisely, the specificity of CRH immunostaining was confirm by co-incubation with 5-fold excess of blocking peptide, while the sections were treated in the same way as described above: the antibody was neutralized by incubation with the blocking peptide; the antibody that was bound to the blocking peptide was no longer available to bind to the epitope present in the protein (Fig. 1). The sections were examined and photographed using a Zeiss Axiovert fluorescence microscope, equipped with a camera and EC Plan-Apochromat.

Pituitary ACTH was localized using the peroxidase-antiperoxidase method. Antiserum to rat ACTH (NIDDK-anti-r ACTH-IC; 1:200) was obtained from Dr. A.F. Parlow, National Hormone Peptide Program, Harbor-UCLA Medical Centre, Carson, CA, USA. The specificity of the antisera was assessed by the National Institute of Diabetes and Digestive and Kidney disease (NIDDK). Endogenous peroxidase activity was blocked by incubation in a hydrogen peroxide solution in methanol. After the blocking procedure and incubation with normal swine serum (1:10; Dako, Glostrup, Denmark) for 1 h, the sections were overlaid with the appropriate dilution of ACTH primary antibodies for 24 h at room temperature. After washing in PBS, the sections were incubated for another 1 h with the secondary antibody (polyclonal swine-anti-rabbit; Dako, Glostrup, Denmark) and again rinsed with PBS. 0.05% 3,3-diaminobenzidine tetrachloride liquid substrate chromogen system was used for antibody localization. Control sections were incubated with rabbit non-immune serum at the same concentration as the primary antibody (in omission of the primary antibody). This resulted in the complete loss of immunoreactivity in the pituitary gland sections (Fig. 1).

Also, pituitary ACTH cells were determined using immunofluorescence. After dewaxing, hydration and rinsing in PBS, the sections were preincubated in normal donkey serum (1:10) for 30 min and then incubated in ACTH primary antibody for 24 h at room temperature. After washing in PBS, the sections were incubated with donkey anti-rabbit Alexa fluor 488 IgG (1:200; Invitrogen) for 2 h, then washed in PBS and coverslipped with mowiol.

2.3. Image analysis

For the evaluation of CRH protein content within ME, measurements of the relative intensity of fluorescent signal (RIF) were performed with Image J, as described previously (Jensen, 2013). Using five immunostained sections from different areas of ME (rostral, three medial and caudal sections) for each animal the RIF for CRH within ME were analyzed. The formula: RIF = Integrated Density – (selected CRH positive area X Mean fluorescence of background readings) was used. The sections were examined and photographed using a Zeiss Axiovert fluorescence microscope, equipped with a camera and EC Plan-Apochromat. So, RIF for CRH content was measured in five sections per animal, followed by calculating the average value per animals, and finally the average per group.

ACTH cell Image analysis: Images were obtained using a confocal