

NOVEL ACUTE STRESSOR EFFECTS ON INTERSCAPULAR BROWN ADIPOSE TISSUE SYMPATHETIC INERVATION AND UCP-1 CONTENT IN CHRONICALLY ISOLATED AND SPONTANEOUSLY HYPERTENSIVE RATS

IVA LAKIC¹, TAMARA DRENCA¹, JELENA DJORDJEVIC¹, P. VUJOVIC¹, N. JASNIC¹,
S. DJURASEVIC¹, SLADJANA DRONJAK-CUCAKOVIC² and GORDANA CVIJIC¹

¹*Institute of Physiology and Biochemistry, University of Belgrade, Faculty of Biology, 11000 Belgrade, Serbia*

²*Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", 11001 Belgrade, Serbia*

Abstract - Interscapular brown adipose tissue (IBAT) is an energy storing organ involved in the maintenance of homeostasis in stress conditions when the balance of energy supplies is disturbed. The major regulator of IBAT activity is the sympathetic nervous system (SNS). Since genetic background is responsible for the individual differences in neuroendocrine stress responsivity, spontaneously hypertensive rats (SHR) that have a genetically increased general sympathetic output are a useful model for studying adaptive processes in stress conditions. Our aim was to test the effect of acute and/or chronic exposure to various stressors (thermal-cold, psychophysical-immobilization and psychosocial-isolation) on IBAT SNS and the metabolic activity in SHR, by measuring the number of monoamine-containing nerve endings and uncoupling protein-1 (UCP-1) content. The obtained results show that the IBAT SNS activity of unstressed SHR was stimulated by the administration of a single acute or chronic stressor and was independent of the duration or type of stressor, while chronic pre-stress of isolation suppressed further the SNS reaction to novel acute stress exposure. The IBAT UCP-1 content followed SNS changes, suggesting that this system is dominant in the regulation of IBAT metabolic rate in SHR.

Key words: Hypertension, SHR, sympathetic innervations, IBAT, stress.

UDC 577.1:591.4

INTRODUCTION

Early studies of Cannon (Cannon, 1929) proposed a concept according to which the sympathetic nervous system (SNS) and adrenal medulla (AM) prepare an animal for the "fight or flight" reaction to stress by raising blood glucose, mobilizing fatty acids from adipose tissue, increasing the heart rate, redistributing blood flow and elevating the metabolic rate. Cannon suggested that the AM is more important than the SNS. However, it has been shown that sympathetic denervation leads to an increase in adipose tissue weight. Nerve stimulation results in fatty acids release and sympathetic or ganglionic blockades inhibits the mobilization of lipid (Gilgen et al., 1962;

Rebuffe-Scrive, 1991). On the other hand, adrenal demedullation is not effective, which suggests that the SNS might be more important than the AM (Rayner, 2001) at least, in the metabolic response to stressors.

The interscapular brown adipose tissue (IBAT) is a mammalian organ specialized for heat production. It functions as a metabolic buffer when energy balance is disturbed, as in states of stress (Himms-Hagen, 1990). Its activity is controlled by complex neural and hormonal factors, with the most important role assumed by the SNS which, through noradrenalin (NA) released from its nerve endings, directly controls IBAT heat production (Girardier and

Seydoux, 1986) by stimulating uncoupling protein-1 (UCP-1) synthesis (Cassard-Doulcier et al., 1993). UCP-1 transforms electrochemical energy into heat (Nicholls and Locke, 1984), enabling small mammals to tolerate cold (Nedergaard et al., 1999) or other conditions when energy homeostasis is disturbed. Our results with 6-hydroxydopamine administration indicate that the intact sympathetic activity is necessary for the maintaining basal level of brown fat UCP-1 (Davidovic et al., 2004). Bearing in mind that sympathetic stimulation also leads to an increase in fatty acid release from white adipose tissue (Rebuffe-Scrive, 1991), we can assume that the SNS is the major regulator of both white and brown fat mobilization to provide homeostasis of energy supplies under conditions of stress.

As far as the influence of genetic factors on adaptive processes is concerned, some differences in individual responses can occur in different animal strains or social paradigms in humans. The genetic background partly contributes to the emergence of individual differences in stress reactivity (Blanchard et al., 1995). The psychoneuroendocrine responses to social stress can also have a genetic origin and therefore the use of SHR can provide an important model for studying adaptive processes (Berton et al., 1998).

SNS has been considered to play an important role in the development of human essential hypertension (Esler et al., 1977, Julius, 1996). Thus the SHR is a widely used model of hypertension in despite the fact that the etiology of this physiological state is not well understood.

Bearing in mind all the above-mentioned, our aim was to test whether animals with a genetically-induced increased general sympathetic output, such as SHR, also have an enhanced local IBAT SNS output and if so, how does it influence the metabolic response of tissue to stress. Special emphasis was on the type (environmental-thermal-cold, psychophysical-immobilization and psychosocial-isolation) and duration (acute and chronic exposure) of stressor and on stressor combinations.

MATERIALS AND METHODS

Experiments were performed on 15 week-old spontaneously hypertensive male rats (SHR). Age-matched normotensive *Wistar* rats (n=6) were used as the control group for determining blood pressure and the catecholaminergic nerve profile density in IBAT. The rats were acclimatized to 22 ± 1 °C, kept at a 12:12 h light-dark cycle, with dark onset at 6 p.m. The animals were given commercial rat food and tap water *ad libitum* and housed two per cage for 15 days before the experiment. The blood pressure of *Wistar* and SHR was monitored one week prior to the experiment, using tail cuff plethysmography of restrained conscious animals. The mean arterial pressure for *Wistar* rats was 100 ± 5 mmHg and for SHR 180 ± 5 mmHg.

The SHR animals were divided into six groups each containing six animals. The **first** SHR group consisted of intact controls. The **second** SHR group was subjected to social isolation for 21-days and killed on the 22nd day. Social isolation was performed by placing one rat in a cage. Visual, acoustic and olfactory communication between isolated rats was reduced to the minimum. The **third** and **fourth** groups were chronically stressed in the same way as the second group (21 days of the isolation) and then on the 22nd day subjected to acute exposure to a novel stressors: cold (6 °C for 2 h) or immobilization (2 h). Immobilization stress was performed according to Kvetnansky and Mikulaj (1970) by fixing all four limbs to a board with adhesive tape. The head was also fixed by a metal loop over the neck area, thus limiting its motion. These animals were killed at the end of exposure to acute stressors. The SHR from the **fifth** and **sixth** groups were exposed to acute stressors, cold (6 °C for 2 h) and immobilization (2 h) respectively, and decapitated after stress termination. Acute stress exposure was exerted inflicted between 8:00 a.m. and 11:00 a.m. to avoid effects of circadian rhythms. All animals were decapitated with a guillotine (Harvard-Apparatus, Holliston, MA, USA).

Animal handling and treatments were carried out in accordance with the Serbian Laboratory Animal

Protection Law and proposed guidelines and protocols that have been approved by the Ethical Committee of Faculty of Biology, University of Belgrade.

Blood was collected from the trunk and IBATs were rapidly excised (4°C) and stored at -70°C. Before freezing, the same part of IBAT was always immediately dipped into frozen-section medium (OKT, Galen-Focus) and later used for determination of the intensity of fluorescent staining of monoamine-containing IBAT nerve profiles according to the method of De la Tore (1980). Five and 10 µm cryostat sections of IBAT were melted onto uncoated glass slides and dipped into a solution containing 2% glyoxylic acid, 10% sucrose, 0.1 M PBS pH 7.4, incubated at room temperature for 10 min and dried under cold airflow. The dried sections were then covered with a drop of glycine-glycerol buffer, heated at 95°C for 2.5 minutes, allowed to cool and cover-slipped. The sections were examined using a BH₂ fluorescence photomicroscope (Olympus, Tokyo, Japan) equipped with excitation filter BP-405 and barrier filter Y-475. Image-J software was used for quantifying the number of noradrenaline-containing nerve fibers per total area.

IBAT UCP-1 levels were estimated by Western blot analysis. Samples of the solubilized mitochondrial fraction (containing 5 µg of the IBAT mitochondrial proteins) were added to an equal volume of buffer (0.125 M Tris-HCl, 0.14 M SDS, 20% glycerol, 0.2 mM dithiothreitol, 0.03 mM bromophenol blue; pH = 6.8). After denaturation by heating to 100°C for 5 min, the samples were separated on a 12.5% polyacrylamide gel and electro-transferred to a PVDF membrane. After removal of nonspecific binding, the membrane was incubated with a solution of rabbit antibody raised against rat UCP-1 (Sigma, U 6382), followed by secondary goat antibodies raised against rabbit immunoglobulin and coupled with horseradish peroxidase (Santa Cruz, goat anti-rabbit IgG-HRP SC-2004). The UCP-1 content was visualized by ECL Western Blotting Detection Reagents (Amersham), by exposure of an X-ray film for 5 min. The intensity of signals was evaluated by the Image Quant program (Molecular Dynamics, Habersham Biosciences). The number of pixels obtained

for the control specimen that had the lowest exposure density represented one arbitrary unit.

Sera were frozen and later used for determination of free fatty acids (FFA) concentrations by the method of Duncombe (1964). The blood glucose level was determined by a glucose analyzer Exac-Tech (MediSense, Cambridge, MA, USA) using Dextrostix reagent strips. Proteins were quantified by the method of Lowry (1951).

The results were expressed as means ± S.E.M. For comparison of differences between groups, one way ANOVA, followed by the Holm-Sidak posterior comparison test were employed with a level of significance set at $p < 0.05$.

RESULTS

The number of fluorescent stains representing the monoamine-containing IBAT sympathetic nerve fibers (40 spots) revealed that in SHR (Fig. 1-a), SNS activity was less pronounced when compared to normotensive *Wistar* rats (110 spots) (Fig. 2). It was also observed that all of the applied stressors provoked changes in the number of IBAT noradrenergic (NA) nerve endings when compared to non-stressed SHR. Figure 1-b shows that chronic isolation stress leads to an increase of fluorescent spots (167), representing increased numbers of monoaminergic nerve profiles. Under the influence of both acute stressors cold (1-c) and immobilization (1-e), the density of nerve endings also increased. The increased density of monoaminergic nerves was significant in chronically stressed SHR that were exposed to acute novel stressors cold (1-d) and immobilization (1-f), only when compared to non-stressed SHR controls (1-a), but not when compared to isolated SHR (1-b) or acutely stressed rats (1-c; 1-e).

Bearing in mind that cold activates adaptive thermogenesis in brown adipose tissue (IBAT) of normotensive rats, the changes of UCP-1 levels as the major metabolic marker of this tissue, were also monitored in SHR (Fig. 3). Our results show that acute cold (2 h) and immobilization (2 h) stress induce an increase

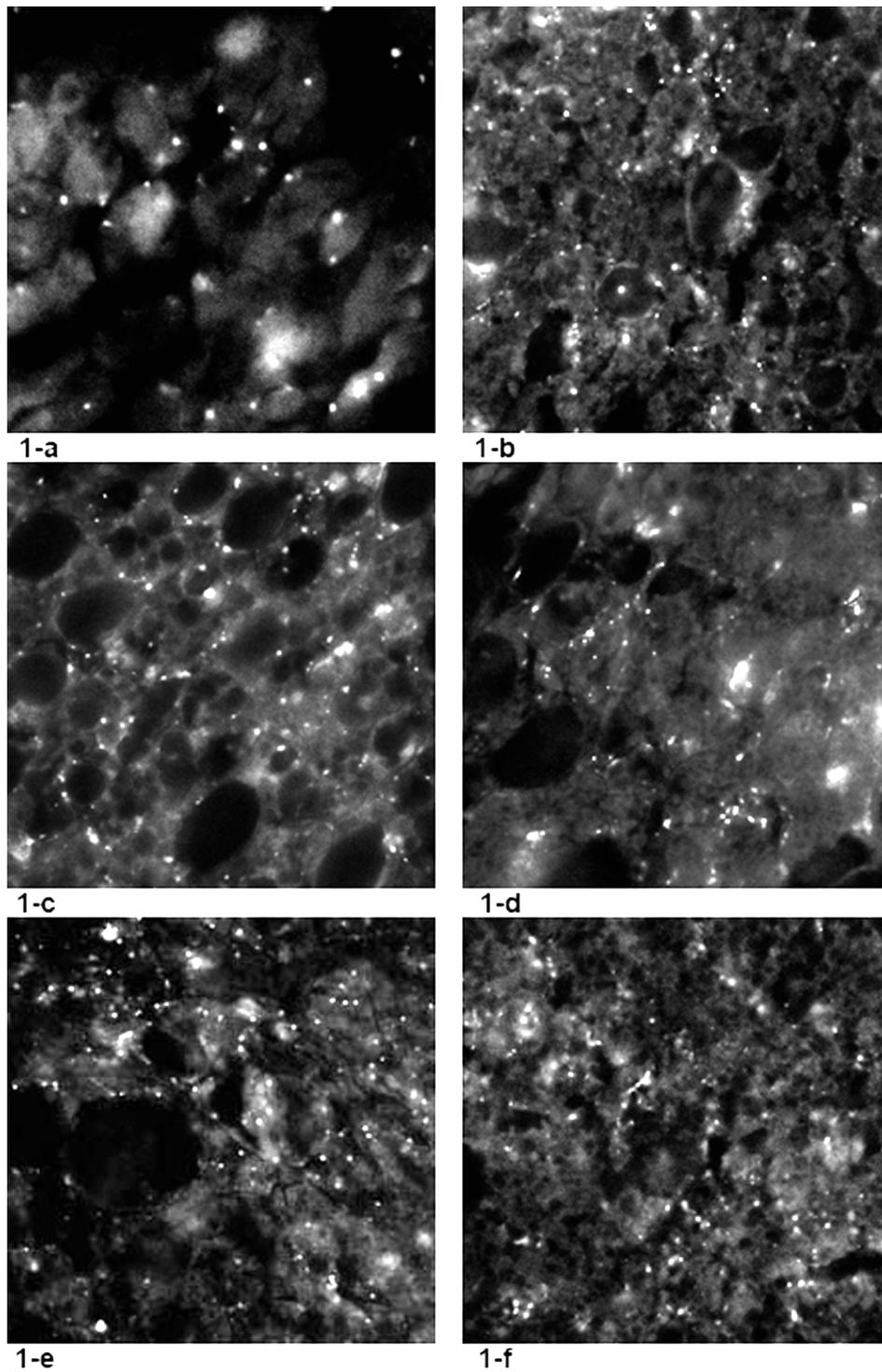


Fig. 1. The density of monoamine containing sympathetic nerve profiles representing as the fluorescent spots in IBAT of SHR, visualised by sucrose-phosphate-glyoxylic acid (SPG) histofluorescence method (De la Torre, 1980). a – control non-stressed rats (40 spots); b – chronic isolation (21 days – 167 spots); c – acute cold (2 h – 162 spots); d – chronic isolation (21 days) + acute cold (2 h) (123 spots); e – acute immobilization (2 h – 214 spots); f – chronic isolation (21 days) + acute immobilization (2 h) (124 spots)

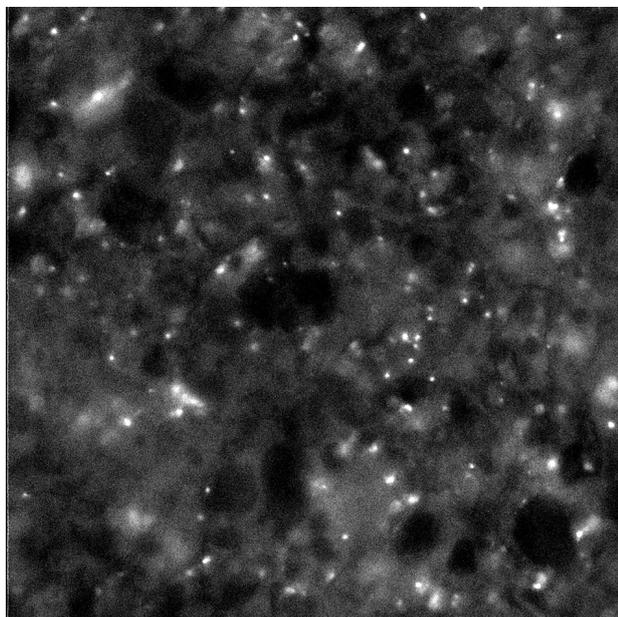


Fig. 2. The density of monoamine containing sympathetic nerve profiles in IBAT of normotensive *Wistar* rats (110 spots), visualised by sucrose-phosphate-glyoxylic acid (SPG) histofluorescence method (De la Torre, 1980)

of the IBAT UCP-1 content that was statistically significant only after the latter stress ($p < 0.001$) in comparison to unstressed SHR controls. The chronic stress of 21 day isolation also increased the IBAT UCP-1 content ($p < 0.05$). However, the IBAT UCP-1 content significantly decreased ($p < 0.05$) when chronically isolated rats were exposed to the acute stress of immobilization, while it remained statistically unchanged when chronically isolated rats were exposed to the acute stress of low environmental temperature.

As a peripheral metabolic marker, blood FFA (Fig. 4) and glucose (Fig. 5) concentrations were determined. The only significant changes in these parameters were observed in SHR exposed to immobilization stress with or without the previous 21-day isolation (Fig. 4: $p < 0.05$, $p < 0.001$; Fig. 5: $p < 0.01$, $p < 0.001$).

As judged by the change in blood ACTH concentration (Fig. 6), all applied stressors, whether chronic

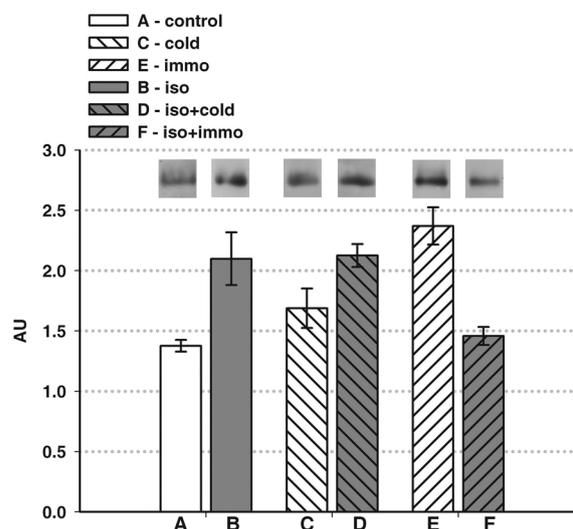


Fig. 3. IBAT UCP-1 content (AU – arbitrary units) in SHR: A – control non-stressed rats; B – chronic isolation (21 days); C – acute cold (2 h); D – chronic isolation (21 days) + acute cold (2 h); E – acute immobilization (2 h); F – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals \pm S.E.M. Statistical significance: A:B - $p < 0.05$; C:D - $p < 0.05$; A:E - $p < 0.001$; B:F - $p < 0.05$.

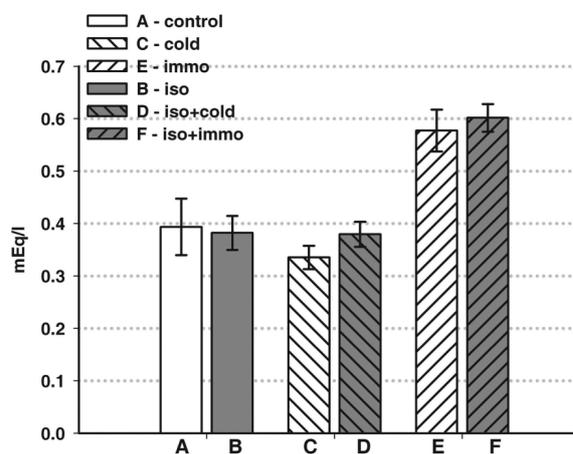


Fig. 4. Serum FFA concentration (mEq/l) in SHR. A – control non-stressed rats; B – chronic isolation (21 days); C – acute cold (2 h); D – chronic isolation (21 days) + acute cold (2 h); E – acute immobilization (2 h); F – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals \pm S.E.M. Statistical significance: A:E - $p < 0.05$; B:F - $p < 0.001$.

(isolation- $p < 0.01$) or acute (cold- $p < 0.01$; immobilization $p < 0.05$), or combined ($p < 0.05$), induced activation of the HPA axis.

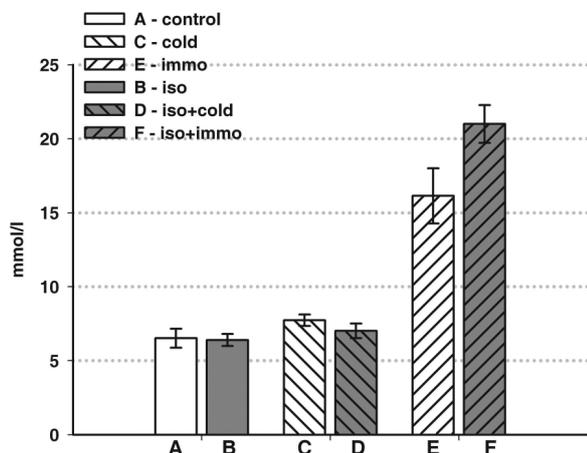


Fig. 5. Blood glucose concentration (mmol/l) in SHR. A – control non-stressed rats; B – chronic isolation (21 days); C – acute cold (2 h); D – chronic isolation (21 days) + acute cold (2 h); E – acute immobilization (2 h); F – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals \pm S.E.M. Statistical significance: E : F - $p < 0.05$; A : E - $p < 0.01$; B : F - $p < 0.001$.

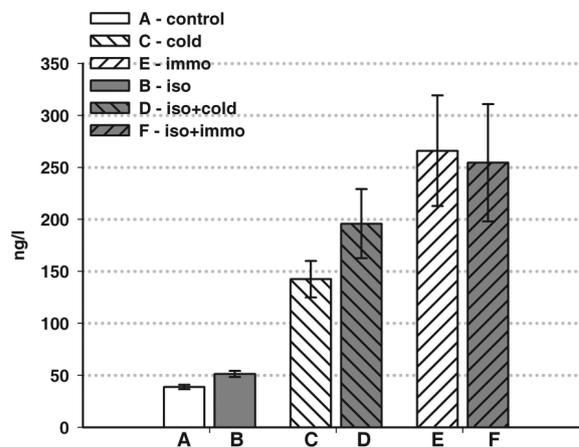


Fig. 6. Plasma ACTH level (ng/l) in SHR. A – control non-stressed rats; B – chronic isolation (21 days); C – acute cold (2 h); D – chronic isolation (21 days) + acute cold (2 h); E – acute immobilization (2 h); F – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals \pm S.E.M. Statistical significance: A : B - $p < 0.01$; A : C - $p < 0.01$; A : E - $p < 0.05$; B : D - $p < 0.05$; B : F - $p < 0.05$.

DISCUSSION

Both white and brown adipose tissues are regarded as organs for energy storage, with the sympathetic nervous system being the major regulator of their activity, which is to maintain homeostasis of the energy supply (Rayner, 2001). In small mammals, the changes in SNS activity are accompanied by changes in IBAT thermogenic capacity induced by NA. The noradrenaline which is secreted from sympathetic nerve endings is the important trophic factor of this tissue that stimulates thermogenesis (Himms-Hagen, 1990; Puigserver et al., 1992; Waldbillig and Desautels, 1992). It was shown that the decrease in the IBAT sympathetic NA turnover is associated with fasting, while an increase occurs in cold exposure or overfeeding (Rayner, 2001). Since SHR exhibits enhanced generalized SNS activity, we hypothesized that these rats might also have an enhanced IBAT sympathetic outflow and consequently could respond differently to applied cold, as well as other stressors of different type and duration. It was shown that these rats react to some environmental stimuli with exaggerated cardiovascular and sympathetic re-

sponses (Chambers et al., 2000). However, our results showed that IBAT SNS activity was less pronounced in unstressed SHR in comparison to normotensive *Wistar* rats. After exposure to cold (2h) SHR IBAT UCP-1, the major metabolic marker of its activity, insignificantly increased. This result differs from the results we obtained in normotensive rats (Cvijic et al., 2004). The photomicrographs demonstrating the number of monoamine-containing nerve profiles in the IBAT of cold exposed SHR showed that there is increased density of SNS nerve endings surrounding the cells. The peripheral metabolic response, judged by the glucose and FFA blood levels, was not significantly changed. Exposure to mobilization (2 h), characterized as a strong psychophysical stressor, also influenced all of these parameters. The density of NA-containing nerve endings, the levels of IBAT UCP-1 and circulating energents glucose and FFA in immobilized SHRs were significantly increased. It is interesting to speculate why immobilization provoked these changes in brown fat since it is not considered a thermal stressor. Intense muscle activity occurred in SHR as they tried to free the fixed limbs and head. It is clear that these processes provoke the

disturbance of energy homeostasis. It was also shown that muscle tissue NA sympathetic innervation is more pronounced in SHR than in normotensive rats (Cabassi et al., 1998).

Judging by the level of circulating ACTH, all of the applied stressors, in addition to activating SNS, also activated the HPA axis. The pronounced quantitative differences depended on the nature of the stress and duration of exposure. Quantitatively, the most pronounced hormonal response was observed in immobilization stress, additionally proving that this stressor is the strongest. Bearing in mind that ACTH secretion is stimulated by hypothalamic CRH, it can be expected that secretion of this hormone is also increased. It was shown that CRH injection into rat brain produces hyperglycemia and that neither hypophysectomy nor adrenalectomy prevent this effect. However, pretreatment with the ganglionic blocker chlorisondamine completely prevents the CRH-induced increase in plasma glucose (Brown et al., 1988). This result suggests that CRH acts in the brain to stimulate sympathetic outflow which results in the development of hyperglycemia. In SHR, the adrenomedullary response to CRH administration is more intense than in normotensive rats (Brown et al., 1988). Therefore, we can assume that in these animals the adrenal medulla is equally involved in the stress response. Our results are consistent with the finding of Dronjak et al. (2004) that after 21 days of isolation, SHR exhibit a significant increase in NA and even a greater increase in circulating adrenaline (A) in response to additional immobilization. These results are in agreement with those of Sohn et al. (2002) who showed that SHR are hyperactive in a novel environment and hyperresponsive to environmental stimuli, exhibiting a pronounced behavioral, sympathetic and cardiovascular responsiveness.

In conclusion, while being less pronounced in SHR than in normotensive *Wistar* rats, IBAT SNS activity was significantly stimulated by acute cold and immobilization stress, as well as by chronic isolation in comparison to unstressed controls. The responses did not depend on the type or duration of stressor.

Exposure to chronic pre-stress suppressed the additional SNS reaction to the novel acute stressor. Since the IBAT UCP-1 contents followed the SNS changes, it can be assumed that this system also assumes a dominant role in the regulation of SHR IBAT metabolic rate.

Acknowledgments - This work was supported by the Ministry of Education and Science of the Republic of Serbia, Grant No 173023.

REFERENCES

- Berton, O., Aguerre, S., Sarrieau, A., Mormede, P., and F. Chaouloff (1998). Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats. *Neuroscience*, **82**, 147-159.
- Blanchard, D. C., Spencer, R. L., Weiss, S. M., Blanchard, R. J., McEwen, B. S., and R. R. Sakai (1995). Visible burrows system as a model of chronic social stress: behavioral and neuroendocrine correlates. *Psychoneuroendocrinology*, **20**, 117-134.
- Brown, M. F., Hauger, R., and L. A. Fisher (1988). Autonomic and cardiovascular effects of corticotrophin-releasing factor in the spontaneously hypertensive rat. *Brain Res.* **441**, 33-40.
- Cabassi, A., Vinci, S., Calzolari, M., Bruschi, G., and A. Borghetti (1998). Regional sympathetic in pre-hypertensive phase of spontaneously hypertensive rats. *Life Sciences*, **62**, 1111-1118.
- Cannon, W.B. (1929). Organization for physiological homeostasis. *Phys. Rev.* **9**, 399-431.
- Cassard-Doulcier, A. M., Gelly, C., Fox, N., Schrementi, J., Raimbault, S., Klaus, S., Forest, C., Bouillaud, F., and D. Ricquier (1993). Tissue-specific and beta-adrenergic regulation of the mitochondrial uncoupling protein gene: control by cis-acting elements in the 5' flanking region. *Mol. Endocrinol.* **7**, 497-506.
- Chambers, J. B., Williams, T. D., Nakamura, A., Henderson, R. P., Overton, J.M., and M. E. Rashotte (2000). Cardiovascular and metabolic responses of hypertensive and normotensive rats to one week of cold exposure. *Am. J. Physiol. Regulatory Comp. Physiol.* **279**, 1486-1494.
- Cvijic, G., Petrovic, N., Djordjevic, J., Davidovic, V., and V. M. Petrovic (2004). Effect of cold exposure on serum DBH and interscapular brown adipose tissue MAO activity in hypothyroid and hyperthyroid T₃- and T₄-treated rats. *Ann. NY Acad. Sci.* **1018**, 214-218.

- Davidovic, V., Petrovic, N., Djordjevic, J., Djurasevic, S., and G. Cvijic (2004). Acute effect of cold on the antioxidant enzymes activities and uncoupling protein-1 content in the brown fat of 6-hydroxydopamine-treated rats. *Journal of Thermal Biology*, **29**, 825-830.
- De la Torre, J. C. (1980). Standardization of the sucrose-potassium phosphate-glyoxylic acid histofluorescence method for tissue monoamines. *Neurosci. Lett.* **17**, 339-340.
- Dronjak, S., Gavrilovic, Lj., Filipovic, D., and B. M. Radojic (2004). Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. *Physiology & Behavior*, **81**, 409-415.
- Duncombe, W. G. (1964). The colorimetric micro-determination of non-esterified fatty acids in plasma. *Clin. Chim. Acta*, **9**, 122-125.
- Esler, M., Julius, S., Zweifler, A., Randall, O., Harburg, E., Gardiner, H., and V. Dequattro (1977). Mild high-renin essential hypertension. Neurogenic human hypertension? *N. Engl. J. Med.* **296**, 405-411.
- Gilgen, A., Maickel, R. P., Nikodijevic, O., and B.B. Brodie (1962). Essential role of catecholamines in the mobilization of free fatty acids and glucose after exposure to cold. *Life Sciences*, **1**, 709-715.
- Girardier, L., Seydoux, J. (1986). Neural control of brown adipose tissue. In: *Brown adipose tissue*. P Thajhurn, DG Nicholas (eds), Edvard Arnold, London.
- Himms-Hagen, J. (1990). Brown adipose tissue thermogenesis: interdisciplinary studies. *Faseb J.* **4**, 2890-2898.
- Julius, S. (1996). The evidence for a pathophysiologic significance of the sympathetic overactivity in hypertension. *Clin. Exp. Hypertens.* **18**, 305-321.
- Kvetnansky, R., and L. Mikulaj (1970). Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology*, **87**, 738-743.
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and R. J. Randall (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
- Nedergaard, J., Matthias, A., Golozubova, V., Jacobsson, A., and B. Cannon (1999). UCP1: the original uncoupling protein – and perhaps the only one? New perspectives on UCP1, UCP2 and UCP3 in the light of the bioenergetics of the UCP1-ablated mice. *J. Bioenerg. Biomembr.* **31**, 475-491.
- Nicholls, D. G., and R. M. Locke (1984). Thermogenic mechanisms in brown fat. *Physiol. Rev.* **64**, 1-64.
- Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B., and J. Nedergaard (1992). Induction and degradation of the uncoupling protein thermogenin in brown adipocytes in vitro and in vivo. Evidence for a rapidly degradable pool. *Biochem. J.* **284**, 393-8.
- Rayner, D. V. (2001). The sympathetic nervous system in white adipose tissue regulation. *Proceedings of the Nutrition Society*, **60**, 357-364.
- Rebuffe-Scrive, M. (1991). Neuroregulation of adipose tissue: molecular and hormonal mechanisms. *International Journal of Obesity*, **15**, 83-86.
- Sohn, H. S., Park, Y. N., and S. R. Lee (2001). Effect of immobilization stress on brain polyamine levels in spontaneously hypertensive and Wistar-Kyoto rats. *Brain Research Bulletin*, **57**, 575-579.
- Waldbillig, D., and M. Desautels (1992). Characterization of norepinephrine-stimulated protein synthesis in rat brown adipocytes. *Am. J. Physiol.* **263**, 1003-1012.