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SINGLE AND COMBINED EFFECTS OF ACUTE AND CHRONIC NON-THERMAL STRESSORS ON RAT INTERSCAPULAR BROWN ADIPOSE TISSUE METABOLIC ACTIVITY

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Abstract - The aim of this study was to examine whether the thermogenic potential of rat interscapular brown adipose tissue (IBAT) changes in response to acute and/or chronic exposure to non-thermal stressors (immobilization and isolation), by measuring the uncoupling protein 1 (UCP-1) content, MAO-A, SOD and CAT activities, as well as the number of IBAT sympathetic noradrenaline-containing nerve fibers. Both acute immobilization (2 h) and chronic isolation (21 days), as well as their combined effects, significantly increased the IBAT UCP-1 content in comparison to non-stressed animals. When applied individually, stressors increased the number of sympathetic fibers in comparison to controls, whereas in combination they decreased it. The activity of IBAT monoamine oxidase-A (MAO-A) decreased under the influence of each stressor independent of its type or duration. SOD activity coincided with MAO-A decrement, whereas CAT activity had an opposite pattern of changes. We conclude that acute and chronic exposure to non-thermal stressors, immobilization and isolation, respectively, affect the metabolic potential of rat IBAT, judging by the increase in UCP-1 content and sympathetic outflow. However, when acute immobilization was applied as a novel stressor to previously chronically isolated animals, an increase in the UCP-1 content was accompanied by a lower IBAT sympathetic outflow, suggesting that IBAT metabolic function under various stress condition is not solely dependent on SNS activity.

Key words: Isolation, immobilization, IBAT, UCP-1, MAO-A, SOD, catalase.

INTRODUCTION

Brown adipose tissue (BAT) is a mammalian organ known to function as a metabolic buffer when the energy balance is disturbed (Himms-Hagen, 1990; Blumberg and Sokoloff, 1997): this primarily occurs during exposure to low ambient temperature or elevated food consumption, but can also be affected by other stressors. In general, stress induces a wide range of physiological reactions involving complex interactions among the nervous, endocrine and immune systems to maintain homeostasis. One of the initial responses to stress is the release of catecholamines from the sympathetic nervous system (SNS) and adrenal medulla. The interscapular brown adipose tissue (IBAT) is one of the major SNS target organs. Sympathetic nerve fibers pass from rostral medullary raphe nuclei and synapse onto postganglionic neurons that innervate brown adipocytes and blood vessels within the IBAT (Girardier and Seydoux, 1986). The axons that innervate adipocytes secrete noradrenaline (NA) which directly controls thermogenesis via the activation of hormone-sensitive lipase (HSL) and uncoupling protein 1 (UCP-1) synthesis (Ricquier and Cassard-Doulcier, 1993). Thus, UCP-1 is the main molecu-

lar marker of IBAT metabolic activity involved in the uncoupling of oxidative phosphorylation and transformation of electrochemical energy into heat (Nicholls and Locke, 1984), the process that enables small mammals to tolerate cold (Nedergaard et al., 1999) and other stress conditions when energy homeostasis is disturbed.

Our previous results have shown that fasting as a metabolic stressor, and heat and cold as thermal stressors, influence rat IBAT metabolic activity by increasing oxygen consumption, thus altering the production of toxic reactive oxygen species (ROS) and consequently the activity of antioxidative enzymes (Cvijic et al., 2000; Djordjevic et al., 2000; Djordjevic et al., 2002). Although the effect of psychosocial stressors when applied alone or in combination with psychophysical stress on the antioxidative enzyme activity in the heart has been described (Djordjevic et al., 2012), only the effect of crowding as a psychosocial stressor, was reported in rat IBAT (Djordjevic et al., 2005).

The aim of the present study was to examine whether the thermogenic potential of IBAT changes during its response to acute and/or chronic exposure to different types of non-thermal stressors (immobilization as a psychophysical, and isolation as a psychosocial stressor), by measuring its UCP-1 content and MAO-A, superoxide dismutase (SOD) and catalase (CAT) activities, as well as the number of IBAT sympathetic monoamine (noradrenaline)-containing nerve fibers., Monoamine oxidase-A (MAO-A) activity was also measured since it is involved in the deamination of monoamines (noradrenaline).

MATERIALS AND METHODS

Animals

Male Wistar 15-week-old rats weighing 320-350 g, were acclimated to 22±1°C and synchronized to a 12 h light/dark regime (lights were turned on at 06:00 h and turned off at 18:00 h). The animals had free access to commercial rat food (Veterinary Institute, Subotica, Serbia) and tap water.

Animal handling and treatments were carried out in accordance with the proposed Serbian Laboratory Animal Protection Law guidelines and protocols approved by the Ethical Committee of Faculty of Biology University of Belgrade.

Experimental design – stress procedures

The animals were divided into four groups, each containing six animals. The first group was the intact control; the second group was subjected to social isolation for 21 days, remaining in a separate room without any visual or auditive connection with other animals, and killed on the 22nd day after the onset of the stressor; the third group was acutely exposed to immobilization stress for 2 h, and the fourth group was kept in isolation for 21 days and then on the 22nd day acutely exposed to a novel stressor – 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 heads were also fixed by a metal loop round the neck to limit their movements. To avoid the effects of circadian rhythms, the rats were exposed to acute stress between 8:00 and 11:00 a.m.

Experimental procedures

All animals were quickly decapitated with a guillotine (Harvard-Apparatus, Holliston, MA, USA). Blood was collected from the trunk, and IBAT rapidly excised (4°C) and stored at -70°C. Before freezing, a part of the IBAT was immediately dipped into frozen section medium (Galen-Fokus) and later used for the staining of IBAT monoamine-containing nerve fibers according to the sucrose phosphate glyoxylic acid method described by de la Torre (1980). After defreezing, IBAT sections (5-10 μm thick) were taken. As BAT and white adipose tissue (WAT) innervation is not homogenous within the fat pad, representative samples across the pad were obtained, weighed, and dipped into a solution containing 2% glyoxylic acid, 10% sucrose, 0.1 M monobasic potassium phosphate (PBS, pH 7.5), and incubated at room temperature for 10 min. IBAT sections were then dried under cold

airflow. To assure a constant treatment protocol we standardized not only the time between IBAT sectioning and section-dipping into glyoxylic acid, but also the drying time and temperature. The sections were then covered with a drop of glycine-glycerol buffer and paraffin oil, heated at 95°C for 2.5 min and immediately afterwards the covers were slipped. All sections were analyzed in the same day to prevent diffusion and/or fluorescence photodecomposition. The sections were examined using a BH2 fluorescence photomicroscope (Olympus, Tokyo, Japan) equipped with exciting filter BP-405 and barrier filter Y-475. The ImageJ software was used for quantifying the number of noradrenaline-containing nerve fibers per total area.

IBAT UCP-1 protein concentration was determined by Western blot analysis. The samples of solubilized mitochondrial fraction (containing 5 μg of IBAT mitochondrial protein) were added to an equal volume of buffer (consisting of 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 electrotransferred to a PVDF membrane. After the transfer of proteins, the membrane was soaked in Tris-buffered saline twice for 5 min, followed by quenching of non-specific binding. After binding of the primary rabbit antibody against rat UCP-1 (1:1000, Alpha Diagnostic International, San Antonio, TX, USA), we used the secondary anti-rabbit antibody coupled with horseradish peroxidase (goat anti-rabbit IgG, 1:5000, Abcam Plc., Cambridge, UK). After washing in TBS, the membrane was incubated with the enhanced chemiluminescence (ECL) plus detection system (Amersham, Buckinghamshire, UK) for 5 min. After draining the excessive ECL plus solution, the immunoreactive bands were detected in a dark chamber. The intensity of signals was evaluated by the Image Quant program (Molecular Dynamics, Amersham Biosciences). The number of pixels obtained for the control group (5 μg protein) represents one arbitrary unit. The UCP-1 content of all groups is expressed relative to control IBAT.

SOD activity was determined by the adrenaline method of Misra and Fridovich (1972) based on the spectrophotometrical measurement of the degree of adrenaline auto-oxidation inhibition by SOD, contained in the examined samples. CAT activity was measured spectrophotometrically by the method of Beutler (1982), based on the rate of hydrogen peroxide degradation by the action of CAT contained in the examined samples. For the determination of MAO-A activity, the IBAT was homogenized in 1 mM potassium phosphate buffer, pH 7.8 and centrifuged at 600 x g for 10 min. Supernatants were used for the radiochemical determination of MAO-A activity according to the method of Callingham and Laverty (1973). Radioactive substrate 3H-5-hydroxy tryptamine was added to samples followed by the extraction of the labeled metabolites into organic solvent for the estimation of the radioactivity in the scintillation counter. MAO-A activity was expressed in nmol per mg of protein per h of incubation (nmol/mg/h). The protein content of the tissue was measured by the method of Lowry et al. (1951).

Data were statistically evaluated by two-way analysis of variance (ANOVA). Holm Sidak comparisons were performed when ANOVA was significant ($p<0.05$). Data are presented as mean ± standard error mean (S.E.M.).

RESULTS

Exposure of rats to non-thermal stressors of a different nature and duration influenced IBAT thermogenic capacity, as judged by the significant changes of its UCP-1 content (Fig. 1). Thus, both acute exposure to immobilization for 2 h and chronic exposure to isolation for 21 days significantly increased the IBAT UCP-1 content in comparison to the control non-stressed animals ($p<0.001$; $p<0.001$). Changes obtained under the influence of chronic isolation as compared to those of acute immobilization, revealed that the former had a significantly stronger effect on IBAT UCP-1 content ($p<0.001$). However, when acute immobilization was applied to previously chronically isolated rats, the UCP-1 content significantly decreased ($p<0.001$) in respect to that

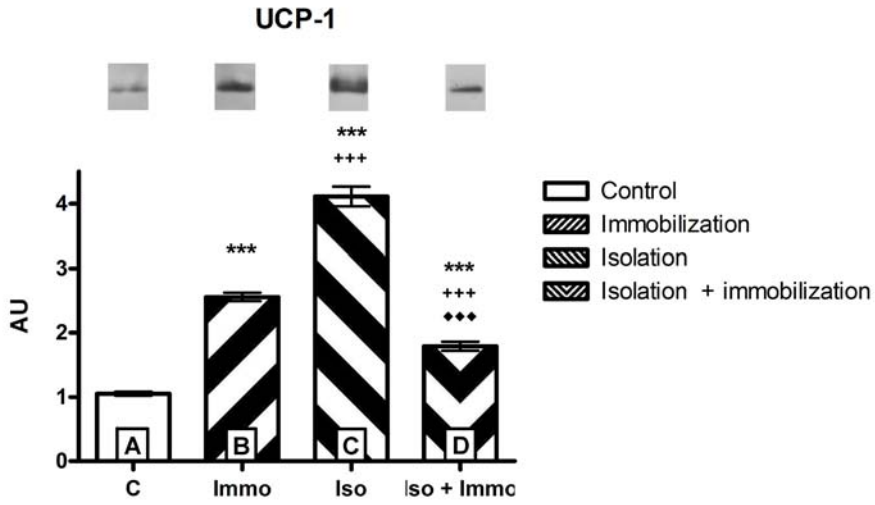

Fig. 1. IBAT UCP-1 content (AU – arbitrary units): A – control non-stressed animals (rats); B – acute immobilization (2 h); C – chronic isolation (21 days); D – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals ±S.E.M. Statistical significance: A:B – $p<0.001$;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

detected after the separate effects of the examined stressors ($p<0.001$; $p<0.001$). However, the UCP-1 content was still significantly above that observed in non-stressed animals ($p<0.001$).

We evaluated the changes in the IBAT sympathetic activity (Fig. 2, Tab. 1) by staining the noradrenaline-containing nerve fibers in the tissue and quantifying their number under the same experimental conditions. Every noradrenaline-containing nerve ending was visible as a bright spot of an IBAT section. When both stressors were separately imposed, the number of noradrenaline-containing nerve fibers increased in comparison to the control animals (B – immobilization, 201.2 ± 8.1; C – isolation, 149.0 ± 7.0; A — controls, 110.8 ± 9.3). However, the combined effect of both stressors decreased the number of sympathetic nerve endings (D – combined effect, 39.3 ± 3.2), which coincides with changes in the UCP-1 content. The number of noradrenaline-containing nerve fibers after the combined effect of applied stressors was far below those obtained in controls (110.8 ± 9.3), whereas under the same conditions the UCP-1 content was still significantly above the control values.

The activity of IBAT MAO-A decreased under the influence of acute immobilization and chronic isolation, applied separately or in combination. Furthermore, after the combined effect of the stressors, MAO-A activity was significantly lower than after independent application of each stressor (Fig. 3a). SOD activity did not change under the acute effect of immobilization but it decreased below the control level after chronic isolation and when the two stressors were applied together (Fig. 3b). On the contrary, CAT activity did not change under acute immobilization but significantly increased ($p<0.001$; $p<0.001$) after chronic isolation and the combined effects of isolation and immobilization in respect to the control rats (Fig 3c).

DISCUSSION

The control systems of IBAT activity were found across the brain with major sympathetic outflow located in the hypothalamic thermoregulatory centers. The hypothalamus directly stimulates brown adipocytes through sympathetic nerves under states of disturbed energy homeostasis, primarily in response to cold. The presented results support the finding that non-thermal stressors, such as isolation and immobilization, can also influence IBAT activity, as judged by the significant elevation of UCP-1 content, the main metabolic marker of this tissue.

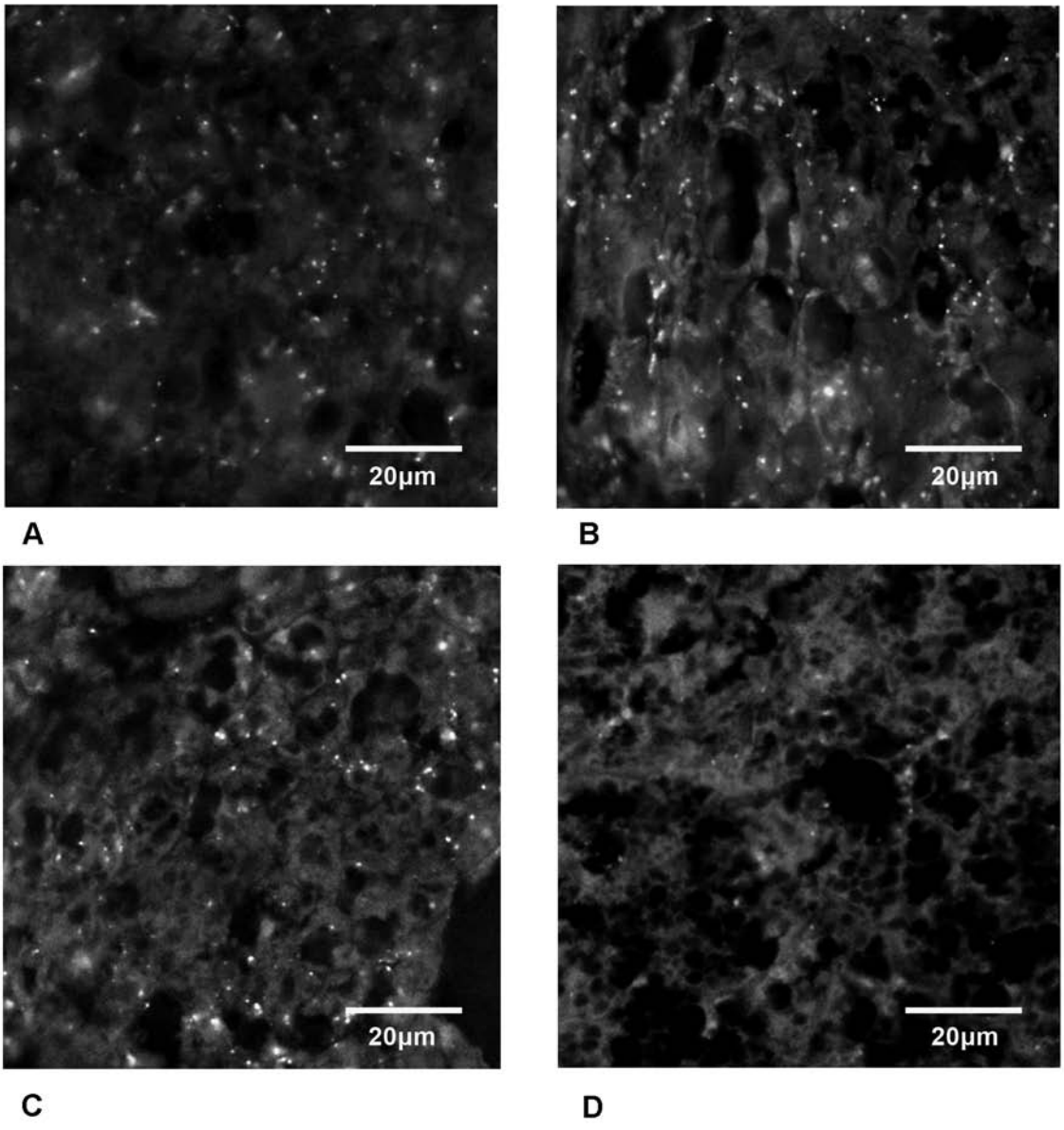

Fig. 2. Photomicrographs demonstrating the density of noradrenaline containing nerve profiles in rat IBAT. A – control non-stressed rats, 110.8 ± 9.3; B – acute immobilization (2 h), 201.2 ± 8.1; C – chronic isolation (21 days), 149.0 ± 7.0; D – chronic isolation (21 days) + acute immobilization (2 h), 39.3 ± 3.2.

This is in agreement with the results of other authors who reported that stressful stimuli, such as restraint, noxious pinching, anxiety paradigm and placement in a novel cage, evoke a constellation of autonomic responses, including an increase in brown fat thermogenesis, supporting a sustained elevation in body temperature – "stress hyperthermia" (Morrison, 2003) and contributing to lower body weight gain through excessive energy loss (Gao et al., 2003). It has been shown that, in the absence of thermogenic stimuli, the sympathetic outflow to IBAT is maintained at a low level by a tonic GABAergic inhibition of IBAT sympathetic hypothalamic neurons. However, our present study showed that the IBAT sympa-

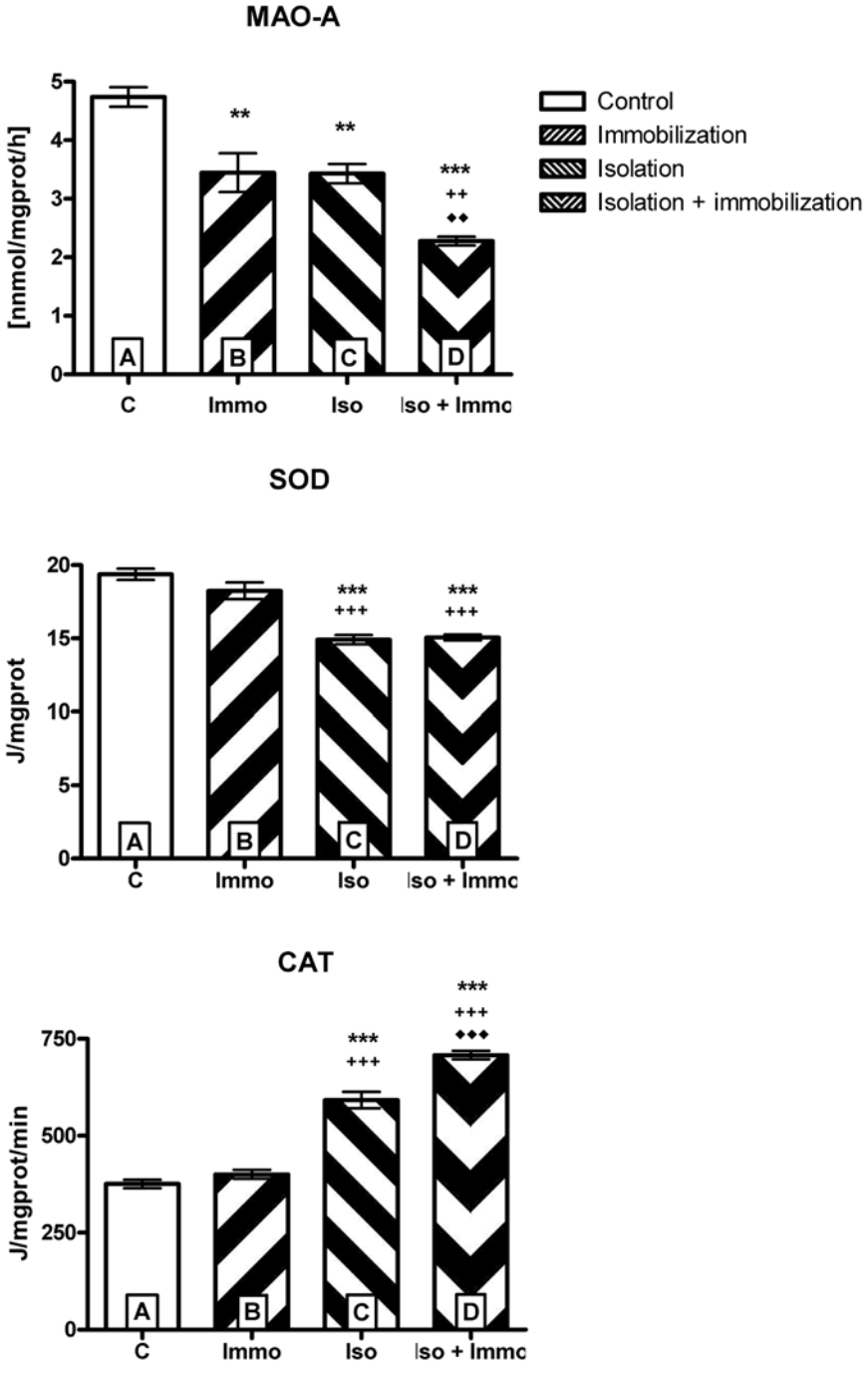

Fig. 3. Activity of MAO-A (nmol/mg prot/h), SOD (J/mg prot) and CAT (J/mg prot/min) in the IBAT. A – control non-stressed animal; B – acute immobilization (2h); C – chronic isolation (21 days); D – chronic isolation (21 days) + acute immobilization (2 h). The values are means of six animals ±S.E.M. Statistical significance: MAO-A: A:B - $p<0.01$; A:C - $p<0.01$; A:D - $p<0.001$; B:D - $p<0.01$; C:D - $p<0.01$; SOD: A:C - $p<0.001$; A:D - $p<0.001$; B:C - $p<0.001$; B:D - $p<0.001$; CAT: A:C - $p<0.001$; A:D - $p<0.001$; B:C - $p<0.001$; B:D - $p<0.001$; C:D - $p<0.001$.

Table 1. Number of the noradrenergic-containing nerve fibers in rat IBAT. The values are presented as the mean ± SEM of at least 6 animals.

Treatment	Control (a)	Acute immobilization (b)	Chronic isolation (c)	Chronic isolation + Acute immobilization (d)
Number of noradrenaline containing nerve fibers	110,8 ± 9,3	201,2 ± 8,1	149,0 ± 7	39,3 ± 3,2
		a:b, $p<0.001$	a:c, $p<0.05$	a:d, $p<0.01$

thetic network, evaluated by the number of stained noradrenaline-containing nerve endings, was additionally activated under the influence of the non-thermal stressors applied. Thus, the acute 2 h exposure of rats to immobilization induced an increase in the number of sympathetic nerve endings in IBAT as compared to the controls. After the chronic psychosocial stressor, the 21-day isolation, the sympathetic nerve endings' number was also increased in respect to the controls. However, when acute immobilization was applied as a novel stressor to previously chronically isolated animals, the number of noradrenaline-containing nerve fibers decreased in comparison to the controls and the rats exclusively exposed to immobilization or isolation. These results indicate that the IBAT UCP-1 content can be changed under the influence of stressors, regardless of the intensity of sympathetic activity, suggesting that UCP-1 synthesis is not only and always dependent on the sympathetic activity. We assume that under these experimental conditions, the IBAT can be also additionally affected by circulating adrenaline originating from the adrenals. Gavrilovic et al. (2005) found changes in the secretion of peripheral adrenaline under the influence of isolation and immobilization stress. It was also reported that when sympathetic activity is depressed, e.g. under the influence of 6-hydroxydopamine, a compensatory acceleration of adrenal medulla functioning, along with an increased release of adrenaline may occur (Takahashi et al., 1993). Djordjevic et al. (2007) confirmed that under the effect of acute immobilization, chronic isolation, as well as under the combined effect of these stressors, the HPA axis activity was also activated, resulting in an increased circulating ACTH level and thus suggesting the elevation of CRH as well. There are data supporting the finding that CRH, the main regulator of ACTH and consequently corticosterone secretion (Brown et al., 1982; Andreis et al., 1991), is involved in the regulation of BAT UCP-1 synthesis (Kotz et al., 2002), as well as in catecholamine secretion (Dunn and Berridge, 1987). Bearing in mind all of the above, we assume that the adrenal medulla was most probably also involved in the regulation of the rat IBAT stress responses, which will be analyzed in our future experiments.

The main fuel for stimulated IBAT thermogenesis is the oxidation of free fatty acids through noradrenaline-induced lipolysis. Thus, during thermogenesis, oxygen utilization increases, with an associated rise in free radical generation in the mitochondria and consequently with changes in free radical-scavenging enzymes. The main enzyme which dismutases superoxide anion radicals into hydrogen peroxide (H_2O_2) is superoxide dismutase (SOD). Catalase (CAT) is also an antioxidant enzyme, localized in peroxisomes of brown adipocytes. It scavenges the H_2O_2 generated either during the dismutation of superoxide anion radicals or in the process of catecholamine deamination by mitochondrial monoamine oxidase (MAO). Our results show that acute immobilization did not alter SOD and catalase activities, while chronic isolation, alone and in combination with acute immobilization, decreased SOD activity and increased catalase activity. However, the activity of MAO-A was decreased regardless of the type and the duration of stressors applied. The strongest decrement of MAO-A activity was observed under the combined effect of the two stressors. At the same time, the increment of UCP-1 content was the weakest as well as the number of noradrenaline-containing nerve endings. The low activity of H_2O_2 producers (MAO-A and SOD) and high activity of the H_2O_2 scavenging enzyme (CAT)

suggest that another source of H_2O_2 is also present, probably the semicarbazide-sensitive amine oxidase (SSAO), present in IBAT, which will be the subject of future experiments. The important factor controlling ROS could be UCP itself (Echtay et al., 2002), namely, it was shown that a mild uncoupling decreases mitochondrial production of ROS (Brand, 2000), and that the superoxide anion increases mitochondrial conductance through effects on UCPs.

In conclusion, our results suggest that non-thermal stressors, acute immobilization (2 h) and chronic isolation (21 days), when applied separately and regardless of their nature and duration, affect rat IBAT metabolic potential, as judged by the observed increment in UCP-1 content. These changes were accompanied by increased IBAT sympathetic activity. When acute immobilization is applied as a novel stressor to previously chronically isolated animals, a decrease in the UCP-1 content is accompanied by lowered IBAT sympathetic activity compared to the sympathetic activity observed after chronic isolation was applied alone. However, this stressor did not bring the UCP content to the level that was registered in non-stressed rats.

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