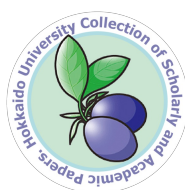




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Author(s)	Teramura, Yasufumi; Terao, Akira; Okada, Yuko et al.
Citation	Japanese Journal of Veterinary Research, 62(3), 117-127
Issue Date	2014-08
DOI	https://doi.org/10.14943/jjvr.62.3.117
Doc URL	https://hdl.handle.net/2115/56895
Type	departmental bulletin paper
File Information	JJVR_62.3 p.117-127.pdf



Organ-specific changes in norepinephrine turnover against various stress conditions in thermoneutral mice

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Received for publication, June 23, 2014; accepted, July 29, 2014

Abstract

The effects of three stressors of different categories, namely cold exposure, immobilization, and lipopolysaccharide (LPS) treatment, on sympathetic nerve activity were examined by assessing its biochemical index norepinephrine (NE) turnover in peripheral organs of C57BL/6 mice. NE turnover was assessed by measuring the decrease in the organ NE concentration 3 h after inhibition of catecholamine biosynthesis with α -methyl-p-tyrosine. NE turnover in brown adipose tissue (BAT) in the room temperature (23°C) control group was as high as that in the cold exposure (4°C) group. Similarly, the mRNA level of the thermogenic marker uncoupling protein 1 (UCP1) in the room temperature control group was as high as that in the cold exposure group. As sympathetic stimulation upregulates the UCP1 mRNA level, we thought that sympathetic nerve tonus in BAT was already accelerated at room temperature. To exclude factors affecting basal sympathetic nerve activity, mice housed at thermoneutral temperature (30°C) were used as controls for the subsequent experiments. In this condition, cold exposure accelerated NE turnover in the BAT, as well as heart and pancreas. The corticosterone level showed a higher trend in the cold exposure group in comparison to the control group. Immobilization accelerated NE turnover in the spleen, pancreas, and white adipose tissue and elevated the corticosterone level. LPS (3 mg/kg, i.p.) did not affect NE turnover in all peripheral organs but elevated the corticosterone level. In summary, the sympathetic nervous and adrenocortical responses to three stressors differed greatly. In particular, sympathetic responses showed clear organ-specific acceleration patterns. This important feature may improve our understanding of the multiplicity of biological responses.

Key Words: sympathetic nervous system, adrenocortical system, cold exposure, immobilization, lipopolysaccharide

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doi: 10.14943/jjvr.62.3.117

Introduction

When animals are exposed to various stressors, a number of changes in both metabolic and immune systems occur depending on the types of stress. For example, acute immobilization and lipopolysaccharide (LPS) treatment in mice result in hyperglycemia and hypoglycemia, respectively [10]. Cold exposure (4°C) and immobilization in mice lead to reduced blood leukocyte and monocyte populations; however, the blood neutrophil population is increased only by restraint stress [2]. To cope with these homeostatic imbalances, the central nervous system evokes physiological responses that ultimately result in the activation of efferent pathways to maintain homeostasis at the systemic level.

The adrenocortical system and the sympathetic nervous system play important roles in responses to stressors. Corticosteroids produced after adrenocortical activation are involved in a wide ranges of physiological processes, including carbohydrate, fat, and protein metabolism, and various important anti-inflammatory actions mediated through the bloodstream. On the other hand, the sympathetic nervous system has the advantage of controlling organ-specific responses [14]. For example, cold exposure accelerates sympathetic nerve activity in the interscapular brown adipose tissue (BAT) as well as in the epididymal and retroperitoneal adipose tissue [8], leading to lipolysis in the adipose tissue and heat production in the BAT [11,17]. Sympathetic nerve activity in the spleen is increased by systemic LPS injection [1], leading to suppression of cytokine production in macrophages [15]. Independent studies using different methods have reported sympathetic nerve responses to cold exposure [13,25], LPS treatment [4], and immobilization [24] in mice; however, no studies have compared the sympathetic nerve responses to various stress types simultaneously under the same environmental conditions.

To evaluate changes in the tonus of

sympathetic nervous activity in various peripheral tissues, norepinephrine (NE) turnover was assessed by measuring the decline in NE concentration after the inhibition of catecholamine biosynthesis with α -methyl-p-tyrosine (α -MT) in peripheral organs, including the heart, spleen, liver, pancreas, BAT, and mesenteric white adipose tissue (WAT) of C57BL/6 mice. The advantage of this method is that it enables evaluation of sympathetic nerve activity in various peripheral organs simultaneously under the unanesthetized condition. Sympathetic nerve responses to cold exposure stress has been assessed using the NE turnover method in Wistar rats [8], Sprague-Dawley rats [26], Syrian hamsters [20], and ICR mice [25] but not in C57BL/6 mice. In this study, we first aimed to optimize the conditions for assessment of NE turnover in C57BL/6 mice and found that it is important to habituate the mice at thermoneutral temperature before cold exposure.

The major objective of the present study was to directly compare the organ specificity of sympathetic nervous responses to different stressor categories under the thermoneutral condition to improve the understanding of the multiplicity of these biological responses. The plasma corticosterone level was also measured to assess the involvement of adrenocortical activation. We selected the following three stress conditions that are widely used and known to affect metabolism and the immune system: cold exposure (environmental stressor), immobilization (psychological stressor), and LPS treatment (infectious stressor).

Materials and methods

Ethics approval of the study protocol: All animal experiments were conducted in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (2011 version; National Institutes of Health, Bethesda, MD, USA). The study protocol was approved by the Animal Care

and Use Committee of Hokkaido University (Sapporo, Japan). All efforts were made to minimize the number of animals used and any pain and discomfort experienced by the subjects.

Animals: A total of 112 male C57BL/6 mice aged 7 weeks were purchased from CLEA Japan (Tokyo, Japan). Mice were housed at three or four animals per cage in an air-conditioned room at $23 \pm 2^\circ\text{C}$ with or without a heating pad ($30 \pm 2^\circ\text{C}$) under a 12-h light/dark cycle (lights on at 8:00 h). Standard laboratory chow (CE-2; CLEA Japan) and distilled water were provided *ad libitum*. After at least 2 weeks of acclimation, mice were subjected to one of the three stress conditions for 3 h starting at 10:00 h: cold exposure (mice were separated into individual polycarbonate cages and placed in a cold chamber at 4°C); restraint stress [mice were individually placed in a small meshed cylinder ($\phi = 3\text{ cm}$, length = 6 cm)]; and LPS injection (3 mg/kg, i.p.). Mice kept at room temperature (23°C) or thermoneutral temperature (30°C) served as controls for the cold exposure study. Mice kept at thermoneutral temperature (30°C) served as controls for the immobilization and LPS injection studies. Phosphate-buffered saline (PBS) was injected into control mice in the LPS injection study.

NE turnover: NE turnover was assessed by measuring the decrease in the organ NE concentration after α -MT injection, as described previously [23]. α -MT inhibits tyrosine hydroxylase and prevents reaccumulation of NE, which is released in response to neural stimuli. The endogenous organ levels then decline at a rate proportional to the initial NE concentration [22]. When the log of NE is plotted against time, the straight line provides the fractional turnover rate (k). Mice were sacrificed by cervical dislocation 0 and 3 h after α -MT injection (300 mg/kg, i.p.), and the heart, spleen, liver, pancreas, BAT, mesenteric WAT, lung, and gastrocnemius muscle were rapidly removed. Organs were weighed,

frozen in liquid nitrogen, and stored at -80°C until the NE assay. Organ samples were homogenized in ice-cold 0.2 M perchloric acid containing dihydroxybenzylamine (Sigma-Aldrich, St Louis, MO, USA) as an internal standard. After the removal of proteins by centrifugation, NE and dihydroxybenzylamine were adsorbed onto activated alumina (Nacalai Tesque, Kyoto, Japan) at pH 8.5, adjusted with Tris-HCl. The alumina was washed with distilled water, and NE and dihydroxybenzylamine were then eluted from it with 0.2 M acetic acid and assayed using an HPLC system with an electrochemical detector (Eicom, Kyoto, Japan).

UCP1 mRNA analysis: Total RNA from BAT was extracted with RNAiso (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. The UCP1 mRNA level was quantitatively measured by real-time RT-PCR and expressed relative to the β -actin mRNA level. In brief, 2 μg of total RNA was reverse-transcribed with an oligo (dT) 15-adaptor primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed on a fluorescence thermal cycler (LightCycler System; Roche Diagnostics, Mannheim, Germany) using SYBR Green I (Roche Diagnostics) as a double-strand DNA-specific dye according to the manufacturer's protocol. Primers used in this study were as follows: 5'-GTG AAG GTC AGA ATG CAA GC-3' and 5'-AGG GCC CCC TTC ATG AGG TC-3' for mouse UCP1 and 5'-TCG TTAC CAC AGG CAT TGT GAT-3' and 5'-TGC TCG AAG TCT AGA GCA AC-3' for mouse β -actin.

Corticosterone assay: Blood samples were obtained 3 h after stress treatments. Plasma corticosterone was measured using the Corticosterone EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analyses: All values are expressed as mean $\pm$ SE. The statistical significances of NE turnover rate, UCP1 mRNA, and plasma corticosterone were assessed by Student's *t*-test;

p values of < 0.05 were considered statistically significant.

Results

NE content in various peripheral organs: As shown in Fig. 1, NE content before stress and α -MT treatment considerably varied among the eight peripheral organs examined, roughly reflecting the density of the sympathetic innervation of each organ. The pancreas (954 ± 29 ng/g organ), heart (767 ± 16 ng/g organ), BAT (717 ± 29 ng/g organ), and spleen (650 ± 31 ng/g organ) had high NE contents, while the lung

(46 ± 2 ng/g organ) and gastrocnemius muscle (26 ± 1 ng/g organ) had low NE contents. In the subsequent NE turnover studies, two organs (lung and gastrocnemius muscle) with NE contents less than 50 ng/g organ were excluded.

Cold exposure study: The NE content exponentially decreased after the inhibition of tyrosine hydroxylase by α -MT. In our initial study, we tried to obtain organ samples from cold-exposed mice 3 and 6 h after α -MT injection. However, because three out of four mice died as a result of hypothermia between 5 and 6 h after α -MT injection, we limited organ sampling to 3 h after α -MT injection. Fig. 2 shows a typical

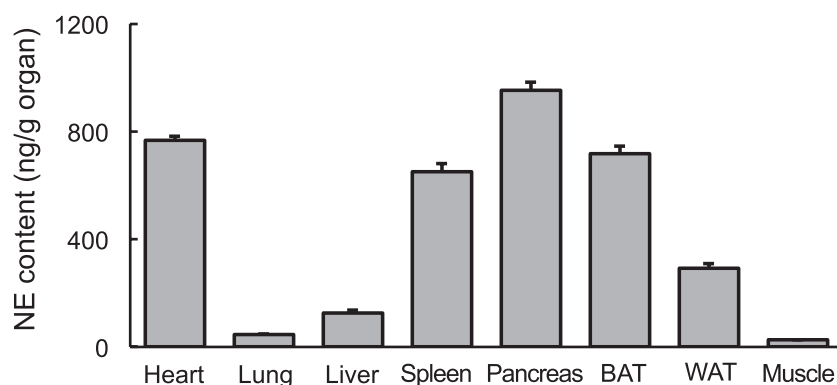


Fig. 1. NE content before α -MT injection in the heart, spleen, liver, pancreas, BAT, WAT, lung, and gastrocnemius muscle. NE values are means $\pm$ SE for seven mice.

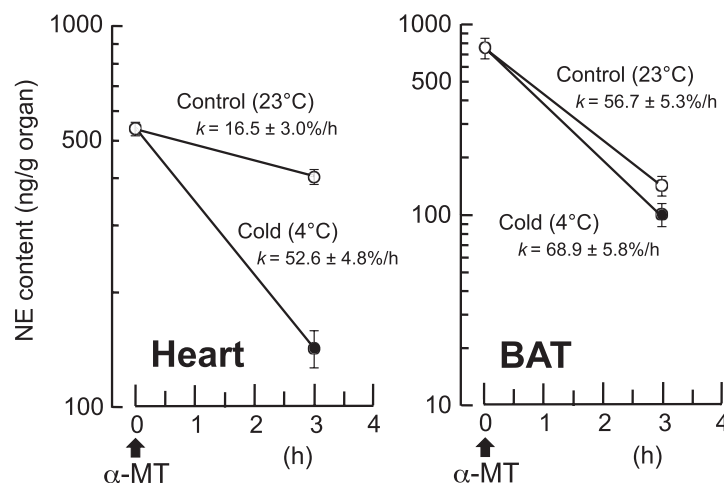


Fig. 2. Semi-logarithmic plots of the decrease in organ NE content in the heart and BAT over time. After inhibition of catecholamine synthesis with α -MT (300 mg/kg, i.p.), mice were placed in a cold chamber (4°C) or kept at room temperature (23°C) for 3 h. Mice not injected with α -MT served as 0-time reference. The fractional turnover rate (k) was calculated from the slope of the fitted line. Data are plotted as means $\pm$ SE for seven mice.

example of NE turnover in the heart and BAT. In the heart, the NE turnover rate assessed in control mice kept at room temperature (23°C) for 3 h was $16.5 \pm 3.0\%/h$, while that assessed in mice kept in cold (4°C) for 3 h was $52.6 \pm 4.8\%/h$. Higher NE turnover was observed in the cold exposure group than in the control group, indicating that sympathetic nerve activity innervating the heart was accelerated by transferring the mice from 23°C to 4°C. On the other hand, the NE turnover rate in BAT in the control group was $56.7 \pm 5.3\%/h$, while that in the cold exposure group was $68.9 \pm 5.8\%/h$. The NE turnover rate in BAT in the control group was as high as that in the cold exposure group.

As sympathetic stimulation upregulates the UCP1 mRNA level, we speculated that sympathetic nerve tonus in BAT was already accelerated at room temperature. Indeed, the UCP1 mRNA level in the control (23°C) group was as high as that in the cold exposure (4°C) group (Fig. 3A). To exclude factors affecting basal energy expenditure, C57BL/6 mice housed at

thermoneutral temperature (30°C) were used as controls for the next cold exposure study. The UCP1 mRNA level in the cold exposure (4°C) group was significantly higher than that in the control (30°C) group (Fig. 3B). Similarly, as shown in Fig. 4, NE turnover was significantly accelerated by cold exposure not only in the heart but also in the pancreas and BAT (Fig. 4). NE turnover in the spleen, liver, and WAT was unchanged. The plasma corticosterone level was also unaffected by cold exposure (Fig. 5A). To keep the control condition constant, control mice were consistently kept at 30°C in the subsequent experiments.

Immobilization study: NE turnover was assessed in immobilized mice under restraint stress. Free-moving mice kept at 30°C served as controls. As shown in Fig. 6, NE turnover in the spleen, pancreas, and WAT was accelerated by immobilization. NE turnover in the heart, liver, and BAT remained unchanged. The plasma corticosterone level was significantly elevated by restraint stress (Fig. 5B).

LPS treatment study: NE turnover was assessed in mice injected with LPS (3 mg/kg, i.p.). PBS-injected mice kept at 30°C served as controls. As shown in Fig. 7, NE turnover in all six peripheral organs assessed was not significantly affected by LPS treatment. The plasma corticosterone level was significantly elevated by LPS injection (Fig. 5C).

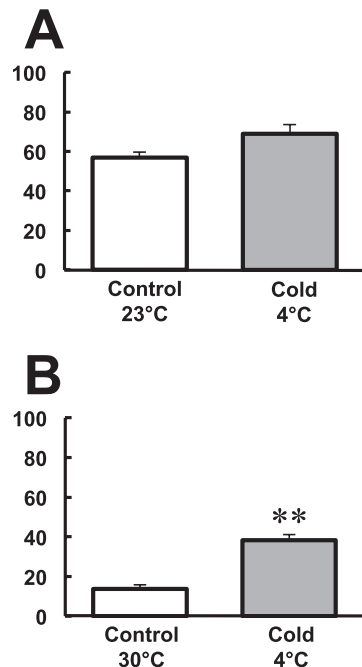


Fig. 3. Effect of cold exposure (4°C) on UCP1 mRNA in BAT. Control groups were kept at 23°C (A) or 30°C (B). UCP1/β-actin values are means $\pm$ SE for seven mice. ** $p < 0.01$ compared with control.

Discussion

In the present study, we first optimized the experimental conditions for detecting the sympathetic nerve responses to cold exposure (4°C) in C57BL/6 mice by housing the animals at a thermoneutral temperature (30°C). Because ambient temperature is a key variable influencing many aspects of mouse physiology, it is important to compare stress responses within the

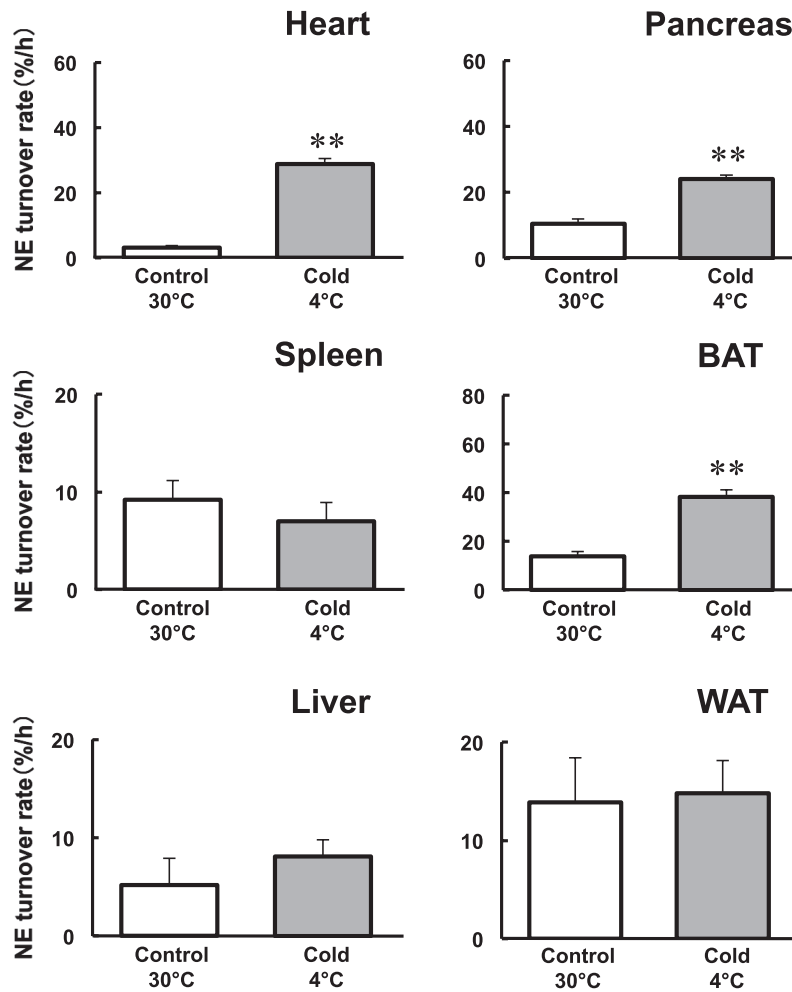


Fig. 4. Effect of cold exposure (4°C) on NE turnover in peripheral organs. Control groups were kept at 30°C. NE turnover values are means $\pm$ SE for 14 mice. ** $p < 0.01$ compared with control.

mouse thermoneutral temperature range. Under this condition, we directly compared the sympathetic nerve response to three stressors of different categories, namely cold exposure, immobilization, and lipopolysaccharide (LPS) treatment. Cold exposure accelerated NE turnover in the heart, BAT, and pancreas, whereas immobilization accelerated that in the spleen, pancreas, and WAT. LPS had no noticeable effect on NE turnover in any of the organs examined. Because it is well accepted that NE turnover in peripheral organs is a biochemical index of sympathetic nerve activity, the present results indicate that a stimulatory action on sympathetic nerves in peripheral organs depends on the type of stress. The advantage of the NE turnover

method is that it enables evaluation of sympathetic nerve activity in various peripheral organs simultaneously under an unanesthetized condition. On the contrary, we must remember the limitations of this method. First, considerable fluctuations in organ NE concentration from individual animals result in variation of the NE turnover rate. Second, it is difficult to detect rather small changes, i.e., statistically insignificant but physiology significant.

In our initial cold exposure study with C57BL/6 mice kept at room temperature (23°C), we were unable to observe a significant increase in either UCP1 mRNA levels or the BAT NE turnover rate in the cold exposure group. In other words, the BAT NE turnover rate in the

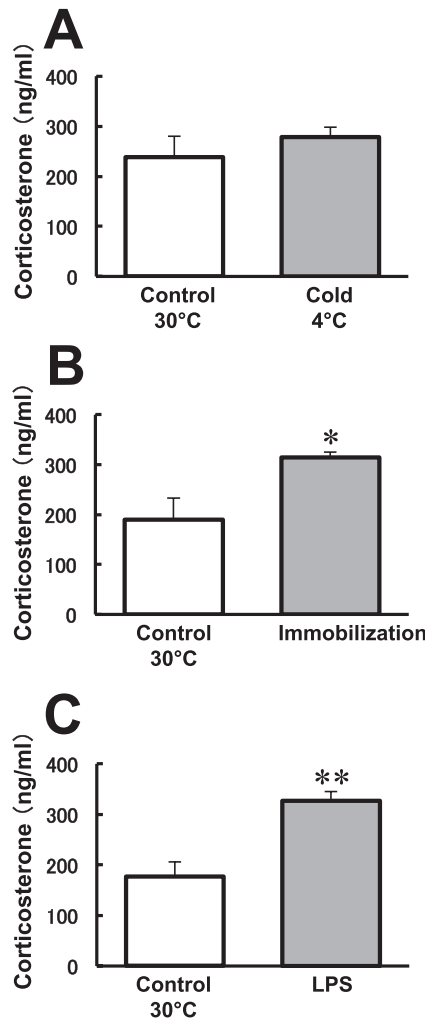


Fig. 5. Effects of cold exposure (4°C), immobilization, and LPS treatment on blood corticosterone levels. Control groups were kept at 30°C. Values are means $\pm$ SE for 7 mice. * $p < 0.05$, ** $p < 0.01$ compared with control.

control group was as high as that observed the cold exposure group. We also evaluated the expression of the thermogenic marker UCP1 in BAT and found that its expression level in the control group (23°C) was as high as that in the cold exposure group. A possible reason why the UCP1 mRNA level and the BAT NE turnover rate were similar between 4°C and 23°C in C57BL/6 mice is that the control temperature was outside the thermoneutral zone. The thermoneutral zone is the region in which basal energy expenditure generates more than sufficient heat to balance heat losses due to the difference

between ambient and body temperatures [5]. Thus, at 23°C, it is possible that C57BL/6 mice are chronically cold-stressed and some degree of UCP1-dependent thermogenesis via increased BAT sympathetic activity likely occurs. To exclude factors affecting basal energy expenditure, C57BL/6 mice housed at a thermoneutral temperature (30°C) were used as controls for further studies.

Acceleration of NE turnover in BAT by cold exposure (4°C–5°C) in comparison with the effects of room temperature (22°C–23°C) was reported in rats [8,26], Syrian hamsters [20], and ICR mice [25]. The experimental design of the ICR mouse study [25] was essentially equivalent to our study as they kept cold-exposed mice at 5°C and control mice at 22°C. Their NE turnover determination protocol was also similar, as both studies measured the decrease in the organ NE concentration after α -MT injection. However, we could not reproduce this phenomenon in our experiment using C57BL/6 mice. Small rodents such as mice have a greater metabolic turnover rate per gram of body weight than humans [12]. This is because smaller animals have a larger body surface area per gram of body weight, which contributes to energy expenditure. Similarly, C57BL/6 mice have significantly lower body weight than ICR mice and may have a higher basal BAT NE turnover rate, which contributes to the high metabolic turnover rate per gram of body weight. In support of this idea, a strong negative relationship between a lower critical temperature of the thermoneutral zone (T_{lc}) and body weight is reported [21]. For example, the predicted T_{lc} of mice weighing 25 g is 28°C, whereas that of mice weighing 40 g is 25.7°C [21]. Because predicted T_{lc} of C57BL/6 mice is higher than that of ICR mice, it is likely that C57BL/6 mice are more susceptible to cold exposure than ICR mice.

NE turnover in the BAT, heart, and pancreas was accelerated by cold exposure. Cold exposure stimulates heat production by means of nonshivering as well as shivering thermogenesis.

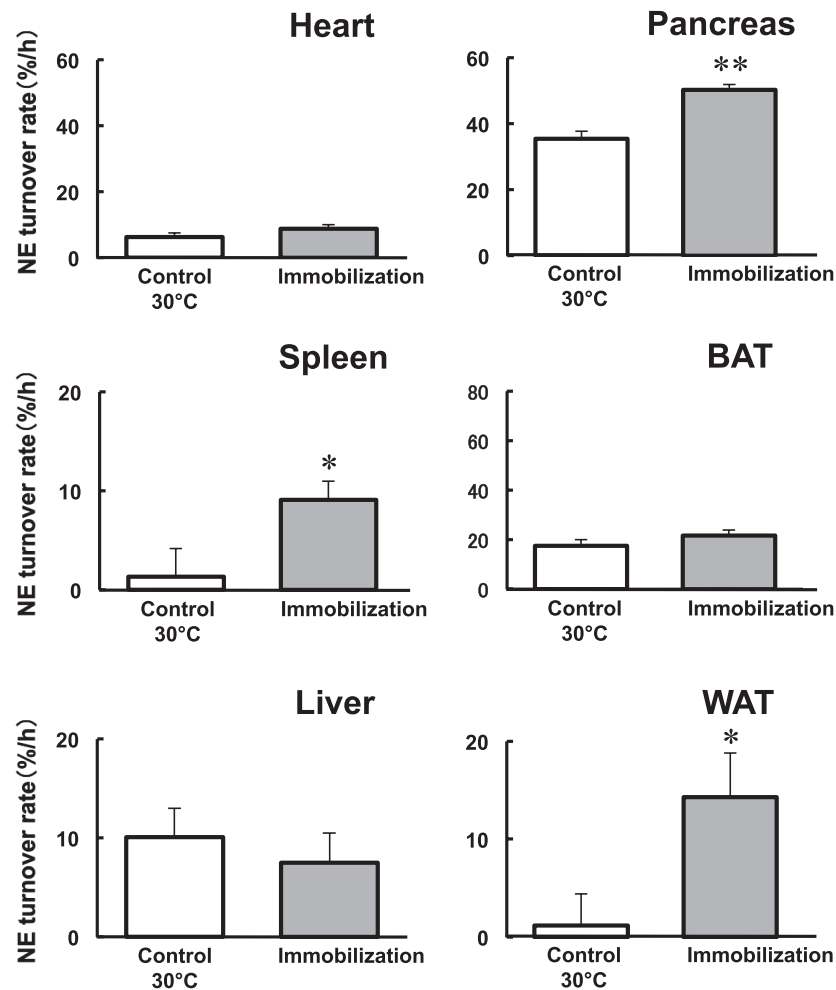


Fig. 6. Effect of immobilization on NE turnover in peripheral organs. Control groups were kept at 30°C. NE turnover values are means $\pm$ SE for 14 mice. * p < 0.05, ** p < 0.01 compared with control.

A number of studies have shown that metabolic acclimation to cold is characterized by enhanced nonshivering thermogenesis, a more efficient means of heat acquisition than shivering [19]. Sympathetic nerve terminals innervating BAT release NE, which activates the thermogenic molecule UCP1 via β 3-adrenergic receptors to produce heat by uncoupling oxidative phosphorylation [19]. Cold exposure is known to effectively activate this pathway to produce heat in BAT as UCP1 knockout mice are sensitive to cold exposure and have a defect in thermoregulation [6]. The heart rate and myocardial contraction are intimately controlled by sympathetic nerves via β 1- and β 2-adrenergic receptors. It has been proposed that in response to cold exposure,

sympathetic nerves innervating the heart facilitate blood circulation, thereby contributing to the distribution of heat produced in BAT. However, in contrast to NE turnover in BAT, NE turnover in the heart was constantly accelerated by cold exposure regardless of the temperature at which control mice were housed, suggesting that the threshold for the sympathetic nerve response for the heart may be lower than that for BAT in C57BL/6 mice. Sympathetic nerves innervating the pancreas inhibit insulin secretion and promote glucagon secretion via α 2- and β 2-adrenergic receptors, respectively, leading to glycogen degradation, gluconeogenesis, and lipolysis. Blood glucose and free fatty acid levels are expected to increase in cold-exposed mice to supply energy

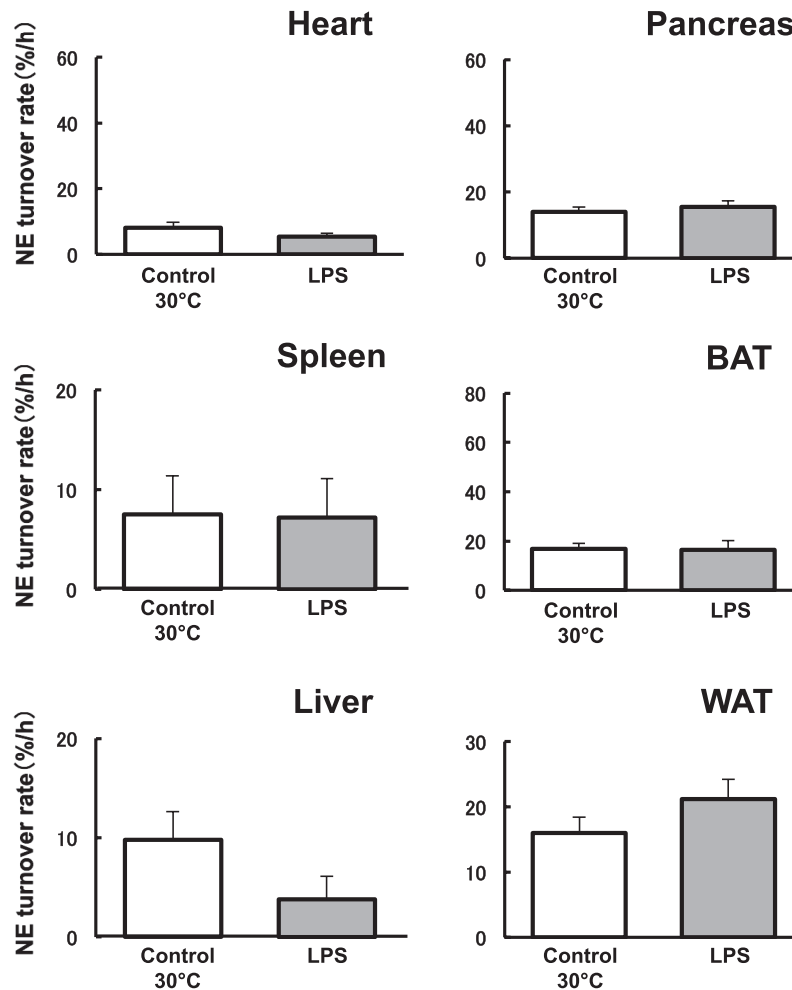


Fig. 7. Effect of LPS treatment (3 mg/kg, i.p.) on NE turnover in peripheral organs. Control groups were kept at 30°C. NE turnover values are means $\pm$ SE for 14 mice.

sources for thermogenesis.

NE turnover in the spleen, pancreas, and WAT was accelerated by immobilization. Splenic sympathetic nerve activation leads to contraction of smooth muscle of the splenic capsule, expelling blood cells into the blood circulation [3]. Abundant sympathetic innervation is also found in the white pulp of the spleen, where T and B lymphocytes are sequestered, and alteration of its activity is known to suppress cytokine production in these cells [15]. LPS-induced cytokine production was reduced in the plasma and brain by immobilization [9]. Similarly, as immobilization is considered a typical psychological stressor [2,10], psychological stress may lead to the suppression of cytokine production by

increasing splenic sympathetic nerve tone. As sympathetic nerves innervating WAT are known to stimulate lipolysis via β 3-adrenergic receptors, blood free fatty acid levels are expected to increase in immobilized mice. The NE content before α -MT treatment roughly reflects the density of sympathetic innervation, and according to this index, mesenteric WAT had much more NE per gram of tissue than epididymal or retroperitoneal WAT [16]. As WAT is a heterogeneous tissue and its characteristics are known to differ among locations, it is speculated that mesenteric WAT, which receives dense sympathetic innervation, may be the major source of lipolysis in WAT.

NE turnover was unaffected in all examined

organs in spite of adrenocortical activation by LPS treatment in this study. Akiyoshi *et al.* [1] reported that LPS accelerated NE turnover in the spleen, lung, liver, and pancreas. One obvious difference between their study and ours is the ambient temperature: 25°C in their study vs. 30°C in our study. In several species, especially small rodents such as mouse, the thermoregulatory response to LPS strongly depends on ambient temperature and a difference of a few degrees centigrade is sufficient to change the response drastically, e.g., from hypothermia to fever [18]. As acceleration of sympathetic activity contribute to heat production associated with endotoxin-induced fever, it is possible that sympathetic responses to LPS may also depend on ambient temperature. Another difference in the NE turnover determination is that we assessed the stress responses in 3 h instead of 6 h [1]. LPS produces a cascade of inflammatory cytokines, beginning with tumor necrosis factor- α (TNF- α), followed by interleukin-1 β (IL-1 β) and then interleukin-6. Indeed, serum TNF- α was detected 45 min after LPS and peaked by 1 h, while IL-1 β was delayed compared with serum TNF and peaked at 4 h [7]. Other important regulatory and effector molecules are produced by macrophages at later intervals, such as IL-12 and interferons [7]. Therefore, it is likely that enough cytokines were not produced to affect the sympathetic nerve tone within 3 h after LPS treatment. It should be noted that in our study, an elevated corticosterone level was observed at a certain time point (3 h after stress); however, NE turnover was assessed during the 3-h frame of stress treatments. The time difference in LPS-induced cytokine production led to delayed sympathetic nervous system activation, which may not be able to result in a significant decline in the NE concentration after the inhibition of catecholamine biosynthesis with α -MT. This interpretation may explain why a significant difference in NE turnover was not observed in any peripheral organs.

In summary, the ambient temperature is

likely to be an important factor to affect mouse physiology. Therefore, comparing stress responses under the same ambient temperature is important. In this condition, the sympathetic nervous system responses to three stressors differed greatly, as indicated by organ-specific NE turnover acceleration patterns. Therefore, the difference in the activation patterns of sympathetic nerve system is clearly dependent on the type of stress. This important finding may improve our understanding of the multiplicity of biological responses, including changes in both metabolic and immune systems, required to maintain homeostasis at a systemic level.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (grant number 22500830) and the Akiyama Foundation.

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