Japanese Journal of Physiology (2003. Jun) 53(3):205-213.

Repeated immobilization stress increases uncoupling protein 1 expression and activity in Wistar rats

Gao, Bihu ; Kikuchi-Utsumi, Kazue ; Ohinata, Hiroshi ; Hashimoto, Masaaki ; Kuroshima, Akihiro

Japanese Journal of Physiology, **53**, 205–213, 2003

Repeated Immobilization Stress Increases Uncoupling Protein 1 Expression and Activity in Wistar Rats

Bihu GAO[1], Kazue KIKUCHI-UTSUMI, Hiroshi OHINATA, Masaaki HASHIMOTO, and Akihiro KUROSHIMA

Department of Physiology 1, Asahikawa Medical University School of Medicine, Asahikawa, 078–8510 Japan

Abstract: Repeat immobilization-stressed rats are leaner and have improved cold tolerance due to enhancement of brown adipose tissue (BAT) thermogenesis. This process likely involves stress-induced sympathetic nervous system activation and adrenocortical hormone release, which dynamically enhances and suppresses uncoupling protein 1 (UCP1) function, respectively. To investigate whether repeated immobilization influences UCP1 thermogenic properties, we assessed UCP1 mRNA, protein expression, and activity (GDP binding) in BAT from immobilization-naive or repeatedly immobilized rats (3 h daily for 4 weeks) and sham operated or adrenalectomized (ADX) rats. UCP1 properties were assessed before (basal) and after exposure to 3 h of acute immobilization. Basal levels of GDP binding and UCP1 expression was significantly increased (140 and 140%) in the repeated immobilized group. Acute immobilization increased GDP binding in both naive (180%) and repeated immobilized groups (220%) without changing UCP1 expression. In ADX rats, basal GDP binding and UCP1 gene expression significantly increased (140 and 110%), and acute immobilization induced further increase. These data demonstrate that repeated immobilization resulted in enhanced UCP1 function, suggesting that enhanced BAT thermogenesis contributes to lower body weight gain through excess energy loss and an improved ability to maintain body temperature during cold exposure. [The Japanese Journal of Physiology 53: 205–213, 2003]

Key words: brown adipose tissue, body temperature, thermogenesis, adrenalectomy; glucocorticoid.

Exposure of rats to stress evokes complex physiological and neurochemical responses that regulate homeostasis [1, 2]. Stress-induced changes in thermoregulation [3, 4] are dependent on activation of the sympathetic nervous system (SNS) and hypothalamic–pituitary–adrenocortical (HPA) axis [5]. We previously demonstrated that repeated exposure of rats to immobilization stress resulted in improved cold tolerance via enhancement of nonshivering thermogenesis (NST) [6].

Brown adipose tissue (BAT) is a major site of NST in small mammals and human neonates and thermogenesis in BAT is an important component of body temperature maintenance and overall energy expenditure [4, 7]. BAT has dense sympathetic innervation and immobilization increases BAT sympathetic nerve activity, and BAT thermal responsiveness was increased in repeatedly immobilized rats [8]. BAT thermogenic activity is regulated by uncoupling protein 1 (UCP1); a mitochondrial protein that uncouples respiration from oxidative phosphorylation so that electrochemical energy can be dissipated as heat [10–12]. In turn, UCP1 activity is dependent on transcriptional regulation [12, 13] and endogenous nucleotide binding state (GDP, ATP etc.) [14].

Stress is also associated with adrenal glucocorticoid secretion that modulates several component of stress responses [15]. Glucocorticoids increase gluconeoge-

Received on April 14, 2003; accepted on May 7, 2003

[1] Present address: Department of Physiology, Arrhenius Loboratories F3, Wenner-Gren Institute, Stockholm University, Stockholm, SE-106 91 Sweden

Correspondence should be addressed to: Masaaki Hashimoto, Department of Physiology 1, Asahikawa Medical University School of Medicine, Midorigaoka-higashi 2–1–1–1, Asahikawa, 078–8510 Japan. Tel: +81–166–68–2321, Fax: +81–166–68–2329, E-mail: mhashi@asahiakwa-med.ac.jp

nesis and lipolysis to increase available substrates for stress responses and promote the vasoconstriction action of catecholamines to prevent heat loss. Further, glucocorticoids promote differentiation of BAT (via BAT glucocorticoid receptors) and expression of UCP1 gene in culture of newborn lamb adipose tissue [16]. While several studies have demonstrated that exogenous glucocorticoids inhibit BAT thermogenesis and UCP1 function *in vivo* [17, 18] and *in vitro* [19, 20], the effects of passive elevation in glucocorticoids differ from the effects of active elevations of glucocorticoids through HPA axis [21, 22]. Further, BAT thermogenesis is regulated by the balance of SNS and HPA axis activation during stress stimuli, however, the role of UCP1 in this process remains unclear. Though it is expected that an increased NST potency in the immobilization-stressed rat is caused by an enhancement of BAT thermogenesis through a control of UCP1 function, there is no experimental result showing changes of UCP1 function under the immobilization stress.

The goal of the present study was to characterize the effect of repeated immobilization on basal level and responsiveness of UCP1 activity and expression in rats. Further, we investigate whether modification of UCP1 function by immobilization is regulated by endogenous corticosterone (glucocorticoids).

MATERIALS AND METHODS

Animals. Male Wistar rats were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and individually housed in wire-bottomed cages at 25±1°C and 50% RH under artificial lighting from 7:00 to 19:00 with free access to standard laboratory chow (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. All rats were habituated to home cages for 1 week before experiments and were handled everyday with or without immobilization throughout the experiments. All immobilization experiments were carried out under the room temperature (25±1°C) slightly lower than the thermoneutral condition [23]. Experiments were performed in accordance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiology Sciences," approved by the Council of the Physiological Society of Japan.

Experiment 1. Sixty-two rats, weighing 180–200 g (aged 7 weeks), were divided into two groups: naive group (naive) and repeated immobilization stress group (repeated). Body weight and food intake were measured everyday. The immobilized stress group was subjected to immobilization for 3 h daily from 10:00 to 13:00 for 4 weeks excluding Sundays. Naive group rates were placed in the same room as immobilized group for the period of immobilization. Immobilization was performed as previously described [24], with slight modification. Briefly, rat forepaws were firmly tied together with adhesive tape; and hind paws were similarly secured. The rat was then laid on its side on a wooden board, and flexible wire gauze was stapled around its contour without restricting breathing. Twenty-four hours after the last treatment, half of the rats from each group were acutely immobilized from 10:00 to 13:00 (acute IMO). The remainder of the animals served as resting controls (resting). After acute IMO, all rats were killed by decapitation without anesthesia.

Experiment 2. Forty-eight rats, weighing 220–250 g (aged 10 weeks), were divided into two groups: a sham operated group (Sham) and an adrenalectomy group (ADX). ADX was performed under thiopental (40 mg/kg body weight) anesthesia. Briefly, a single dorsal midline incision was made through the skin at the level of the kidneys. After retraction of the skin to the left, a longitudinal incision was made along the lateral border of the dorsal muscle mass. The fat tissue lying between the adrenal and the kidney was held with fine-toothed curved forceps and pulled into the incision so that the adrenal was visualized for removal. The second adrenal was removed in a similar fashion. Control groups were sham operated in an identical manner, excluding actual adrenal removal. After surgery, ADX rats were provided with physiological saline to drink ad libitum. Forty-eight hours after surgery, half of the rats from each group were acutely immobilized from 10:00 to 13:00 (acute IMO), and rats were killed by decapitation without anesthesia.

In both experiments, interscapular BAT (iBAT) was excised, and a small portion was immediately frozen in liquid nitrogen and stored at −70°C for Northern blot analysis. Mitochondria were isolated from the remainder of the tissue for GDP binding and Western blot analysis.

Isolation of BAT mitochondria. Mitochondria were isolated from pooled iBAT from 2–3 rats according to the method of Cannon *et al.* [25], Briefly, the tissue was homogenized in a 250 mM sucrose solution, filtered through gauze and centrifuged at 8,500×*g* for 10 min. The pellet was resuspended in the sucrose solution and centrifuged at 700×*g* for 10 min. The supernatant was centrifuged at 8,500×*g*. The resulting mitochondrial pellet was resuspended in 250 mM sucrose solution with 2% fatty-acid-free bovine serum albumin and centrifuged at 8,500×*g*. The pellet was

again resuspended in the sucrose solution and centrifuged at 8,500×*g*. The resulting albumin-washed mitochondria was suspended in a small volume of the sucrose solution and stored on ice. All steps were carried out within 2 h and while on ice. Mitochondrial protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and immediately used for GDP binding experiments or stored at −70°C for Western blot analysis.

[^{3}H]GDP binding experiment. The amount of GDP binding to mitocondria was used as an index of UCP1 activity [26]. Briefly, the mitochondria were incubated at 21°C for 10 min at a concentration of 1 mg mitochondrial protein/ml in a medium consisting of 100 mM sucrose, 20 mM K-TES, 1 mM EDTA, 5 μM rotenone, 10 μM GDP (Sigma, St. Louis, USA) labeled with 0.27 μCi/ml [^{3}H]GDP (Amersham Pharmacia Biotech, England, UK). Next, 0.135 μCi/ml [^{14}C]sucrose (Amersham Pharmacia Biotech) was added as a marker of the extra mitochondrial volume. After incubation, the entire incubation medium was centrifuged at 8,500×*g* for 3 min. The resulting pellet was fully dissolved in scintillation medium consisting of 0.5% PPO and 17% H_2O in Triton X-100/toluene (1 : 2, vol/vol) and transferred to a scintillation vial. The incubation tube was washed twice more with the scintillation medium. ^{3}H and ^{14}C was counted on Wallac 1414 DSA liquid scintillation counter (WinSpectral α/β and Guardian, USA). The specific GDP binding was calculated as the excess amount of non-bound [^{3}H]GDP found in extra mitochondrial volume (measured by [^{14}C]sucrose).

cDNA probe preparation. Total RNA of iBAT was prepared using TRIzol Regent (Gibco BRL, Life Technologies, Inc., Frederick, USA). UCP1 cDNA probe was obtained by RT-PCR using iBAT total RNA as template. Primers for the UCP1 cDNA fragment were 5′-GGATCAAACCCCGCTACACTG-3′ (forward, position 625–645) and 5′-CAGGATCCGAGTCGCAGAAAA-3′ (reverse, position 1001–1021) based on the rat UCP1 cDNA sequence (M11814, GenBank). Thirty cycles of RT-PCR was carried out with Titan One Tube RT-PCR System (Roche, Germany) under the following conditions: reverse transcription at 50°C for 30 min; denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 68°C for 1 min. The amplification products were analyzed and purified from electrophoresis agarose gel using High Pure PCR Product Purification Kit (Roche, Germany). Probes were labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) by random primer labeling using cDNA labeling beads (Amersham Pharmacia Biotech). A 35-base synthetic oligonucleotide specific for the 18S rRNA subunit was used for correction for loading and blotting differences. The oligonucleotide was labeled with [^{32}P]ATP (Amersham Pharmacia Biotech) using Polynucleotide Kinase (Pharmacia Biotech, USA).

Northern blotting. The iBAT total RNA (15 μg) was electrophoresed in a 1.25% agarose gel containing 6.2% formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) by capillary blotting. The membrane was hybridized for 1 h at 65°C in QuikHyb Hybridization Solution (STRATAGENE, La Jolla, CA, USA) with labeled probe, then washed twice for 15 min in 2×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% (wt/vol) SDS at room temperature and once in 0.1×SSC/0.1% SDS at 60°C for 30 min. The membrane was exposed to an imaging plate, and the autoradiogram signals were quantified with a BAS2000 Bioimage analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis. UCP1 protein expression of in BAT mitochondria from the rats in experiment 1 was determined. The iBAT mitochondrial protein (2.0 μg) was boiled in sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, and 6% β-mercaptoethanol), subjected to Bis-Tris Gels (NOVEX Electrophoresis System, CA, USA) for electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech, England) using a standard procedure. The membrane was incubated with an affinity-purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of mouse UCP1 (1 : 4,000 dilution, Santa Cruz Biotechnology, Inc., USA) as primary antibody and a peroxidase-conjugated affinity-purified anti-goat donkey IgG (1 : 20000 dilution, Rockland, USA) as secondary antibody. Detected protein was visualized using ECL Western blotting detection reagents and Hyperfilm ECL (Amersham Pharmacia Biotech). Signal analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Measurement of plasma corticosterone. In experiment 2.5 ml of trunk blood was collected into tubes containing 100 μl of heparin immediately after rats were decapitated. The blood was then centrifuge (3,000 rpm, 10 min), and plasma was collected into small plastic tubes and stored at 20°C. Plasma corticosterone levels were measured by RIA using the Rat corticosterone [^{125}I] assay system with Amerlex TM-

Table 1. Effect of repeated immobilization on body weight, food intake, and core temperature in rats.

	Naive		Repeated	
	Resting	Acute IMO	Resting	Acute IMO
Body weight (g) at experiment	307.0±3.8	303.0±4.7	233.0±2.3*	232.0±3.5*
Food intake (g/d/100 g body weight)	6.0±0.1	6.0±0.1	6.0±0.1	6.0±0.1
Core temperature (°C)	37.00±0.07	38.60±0.12*	36.80±0.07	38.40±0.18#

The temperature was measured immediately after decapitation by inserting a thermistor probe into the rectum. Naive: control rats without repeated immobilization; Repeated: rats that were immobilized 3 h daily for 4 weeks. Resting: rats without 3 h immobilization. Acute IMO: rats exposed to 3 h immobilization on the last experiment day. Values show the means±SEM. * $p<0.001$ vs. resting in naive group; # $p<0.001$ vs. resting in repeated group.

M magnetic separation (Amersham LIFE SCIENCE, Buckinghamshire, UK).

Core temperature measurements. Immediately after rats were decapitated, core temperature was measured using a thermistor probe inserted 5 cm into the rectum. We had confirmed in the previous pilot experiment that the core temperature at this moment did not differ from that before decapitation.

Statistical analysis. Data are presented as the mean±SEM, and ANOVA was performed using StatView software (SAS, Cary, NC, USA). The magnitude of the increase in GDP binding was assessed by 95% confidence interval.

RESULTS

Experiment 1

Effect of immobilization on body weight and food intake. Body weights were averaged through the last 3 d of experiment. There was no difference in initial body weight and food intake among the groups. As shown in Table 1, repeated immobilization-stressed rats gained 20% less body weight ($p<0.001$) than control naive rats without a difference in food intake (per 100 g body weight).

Effect of immobilization on BAT UCP1 expression. Basal UCP1 mRNA in the repeated immobilization group was 140% of the basal UCP1 mRNA in naive rats (Fig. 1; $p<0.001$). In contrast, acute immobilization did not affect UCP1 gene expression level in either group (Fig. 1B). Results of Western blot analysis demonstrated a change in UCP1 protein expression that paralleled changes in UCP1 mRNA (Fig. 1C).

Effect of immobilization on mitochondrial GDP binding in BAT. Figure 2A shows the changes in BAT mitochondrial GDP binding in naive and repeatedly immobilized rats. The resting level of GDP binding in naive rats was 198.3±9.1 pmol/mg of mitochondrial protein. The repeated immobilization

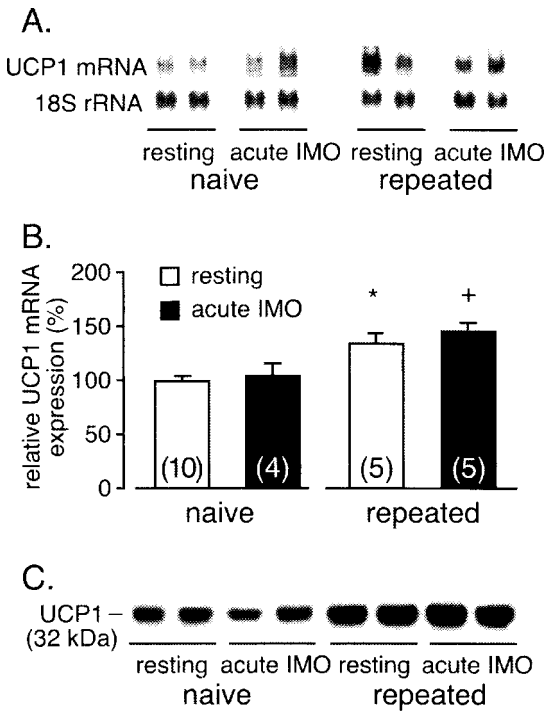

Fig. 1. Effects of immobilization on BAT UCP1 expression in rats. Northern blot analysis for UCP1 mRNA with representative UCP1 mRNA and 18S ribosomal RNA (rRNA) signals (**A**) and quantification (**B**) are shown. Relative levels of UCP1 mRNA expression were quantified and normalized using the corresponding 18S rRNA levels. Values, expressed as a percentage of UCP1 mRNA expression of the resting level in naive rats, are represented as mean±SEM. Resting: rats without 3 h immobilization (open column); Acute IMO: rats exposed to 3 h immobilization on the last experiment day (closed column). Sample numbers are shown in parenthesis in each column in this and the following figures. Representative images of Western blots analysis of rat UCP1 protein in BAT mitochondria are shown in C. Interscapular BAT mitochondrial protein was resolved by 10% SDS-PAGE. * $p<0.001$ vs. resting, naive group; + $p<0.001$ vs. acute IMO, naive group by ANOVA. All symbols in the following figures express the same mean as shown in this.

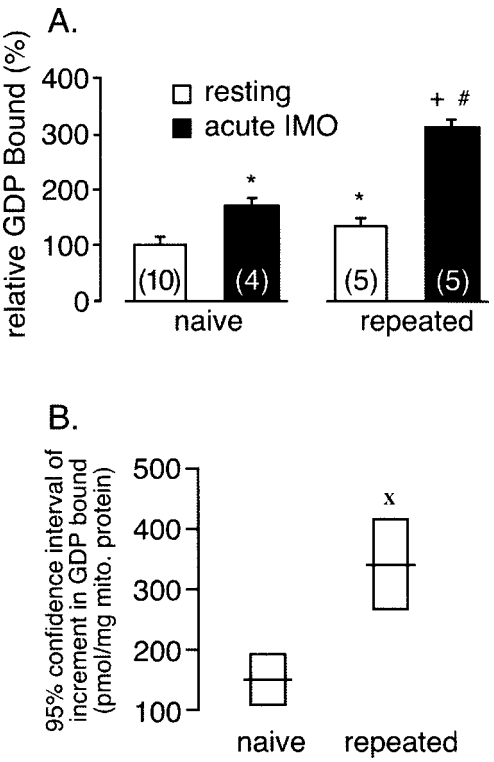

Fig. 2. Effects of immobilization on UCP1 activity in rats. **A**: Effects of immobilization on BAT mitochondrial GDP binding in rats. **B**: Acute immobilization-induced GDP binding increment in naive and repeated immobilization groups. Values are represented as mean±SEM.; $^{\#}p<0.001$ vs. resting, repeated immobilization group; $^{X}p<0.05$ *vs.* GDP binding increment in naïve, acute IMO group.

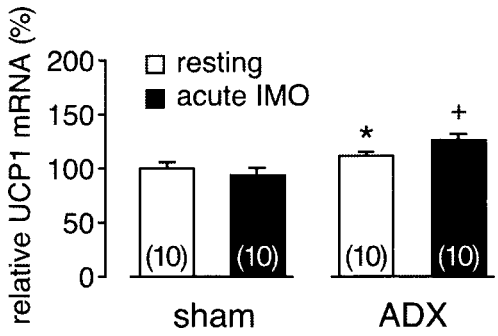

Fig. 3. Effects of immobilization on BAT UCP1 expression in ADX rats. Relative levels of UCP1 mRNA expression were quantified and normalized using the corresponding 18S rRNA levels. Values, expressed as a percentage of UCP1 mRNA expression of the resting level in sham operation rats, are represented as mean±SEM. $^{*}p<0.001$ vs. resting, sham operated group; $^{+}p<0.001$ *vs.* acute IMO in sham operated group by ANOVA.

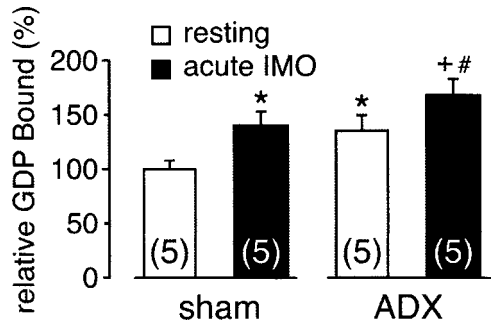

Fig. 4. Effects of immobilization on BAT mitochondrial GDP binding in ADX rats. Values are represented as mean±SEM (sample numbers are shown in parenthesis in each column). Sham indicated sham operated rats, while ADX indicates adrenalectomized rats. Symbols except for $^{\#}p<0.001$ *vs.* resting in ADX group mean the same as in Fig. 3.

group showed a 40% increase above this level ($p<0.001$). In response to acute immobilization, GDP binding of naive rats increased up to 180% ($p<0.001$) of naive-group's resting level, while repeated immobilization rats increased to 220% ($p<0.001$) of repeated-group's resting level. The acute immobilization-induced increment in GDP binding was significantly greater in repeatedly immobilized groups (338.0±73.5 pmol/mg mitochondrial protein) than in naive control group (152.9±40.9 pmol/mg mitochondrial protein) ($p<0.05$), as shown in Fig. 2B.

Effect of immobilization on core temperature. In the resting condition, core temperatures of naive rats and repetitive immobilization were similar, ranging from 36.8–37.2°C ($n=5$). Acute immobilization resulted in temperature increases to 38.3–39.0°C in naive groups ($p<0.001$) and 38.2–38.8°C in repeated immobilization groups ($p<0.001$). However, this difference did not reach statistical significance (Table 1).

Experiment 2

Effect of ADX on UCP1 expression during immobilization. Acute immobilization had no effect on UCP1 mRNA expression in Sham group rats (Fig. 3). Resting level UCP1 expression was significantly higher in ADX group than in Sham group ($p<0.001$). UCP1 expression was significantly increased by acute IMO ($p<0.001$) (Fig. 3).

Effect of ADX on BAT mitochondrial GDP binding during immobilization. ADX resulted in significantly increased GDP binding ($p<0.01$). Acute IMO resulted in significantly increased GDP binding in Sham group ($p<0.05$) and ADX group ($p<0.05$). GDP binding in ADX+acute IMO was significantly greater than that in Sham+acute IMO ($p<0.05$) (Fig. 4).

Effect of ADX on core temperature during immobilization. In the resting condition, core temperatures were 36.5–37.2°C ($n=10$) in Sham rats and 36.7–37.2°C ($n=10$) in ADX rats. Acute immobi-

Table 2. Plasma corticosterone level and core temperature in response to immobilization stress in ADX rats.

	Sham		ADX	
	Resting	Acute IMO	Resting	Acute IMO
Corticosterone (ng/ml)	13.40±1.61	21.40±3.78*	0.10±0.0*	0.10±0.01*
Core temperature (°C)	36.70±0.08	37.80±0.09*	37.00±0.05	37.20±0.20

Values are represented as mean±SEM. * $p<0.001$ vs. resting in Sham group.

lization resulted in temperature increases of approximately 1.2°C in Sham groups ($p<0.001$), but had no effect on core temperature in ADX rats after acute IMO (Table 2).

Plasma corticosterone. Post-operative plasma corticosterone level was 5.73–20.24 ng/ml ($n=10$) in sham group rats, while was reduced down to 0.10–0.12 ng/ml in ADX rats (Table 2). Acute immobilization resulted in a 60% increase in sham group plasma corticosterone level, but had no effect on ADX group plasma corticosterone level.

DISCUSSION

The present study demonstrated that repeated exposure of rats to immobilization stress increased activity and expression of UCP1. This may contribute to enhanced BAT thermogenesis and reduced rate of body weight gain in repeatedly immobilized rats. In contrast, acute immobilization produced an increase in GDP binding but did not affect UCP1 expression. Exposure of ADX rats to acute stress resulted in increased UCP1 expression, suggesting that acute change in HPA-axis factors including corticosterone elevation might inhibit UCP1 expression *in vivo*.

Under the present experimental conditions without fasting, ulcer-like changes of the gastric intima were not observed (data not shown), which agrees with previous report of experimental ulceration in the rat [24]. This fact may exclude a possibility that this pathological trouble due to the immobilization stress reduced the body weight gain in stress-repeated group. Body mass composition was not measured in the present experiment, however, white fat mass decrease and BAT mass increase might contribute to the weight difference between naive- and repeated [6]. Further, the small body weight in the repeated group seems to be somehow regulated since food intake per unit body weight of repeated-group rats did not differ from that of naive-group rats (Table 1). Substances, for instance leptin and thyroid hormone, relating to energy balance in the body might be involved in the mechanism of inducing body weight difference between control and repeated immobilization groups in the present experiments. Since these two substances are well known as energy expenditure stimulants and food intake inhibitors in most mammalian species including rats, if the repeated immobilization would increase the plasma level of them, it could be resulted in a decreased body weight gain. It is, however, unlikely, because leptin mRNA level was decreased in the repeated immobilization group at 1 week after beginning of repetition (data not shown), and which agrees with the findings of Harris and co-workers [27]. Similarly, thyroid hormone level in blood plasma did not change just after acute immobilization (data not shown) as well as its decrease by repeated immobilization stress [28].

UCP1 is a principal mediator of adaptive NST and metabolic inefficiency [29]. A significant role of UCP1 in thermogenesis is demonstrated, as genetic ablation of UCP1 in mice results in a relative loss of thermogenic capacity [30]. In the present study, 3 h of immobilization increased GDP binding in naive rats and repeatedly immobilized rats, with a greater in repeatedly immobilized rats. Although the exact mechanisms that regulate UCP1 activity remain controversial [10], GDP binding is accepted as a sufficient indicator of UCP1 activity [31], and NST capacity is determined by the total amount of UCP1 in a small mammal. An enhancement of UCP1 expression is observed repeatedly immobilized rats and is associated with BAT hyperplasia and development of dense mitochondrial cristae [6], which results in enhancement of thermogenic capacity and improved tolerance to cold exposure. The notion of a differential effect of repeated and acute immobilization on UCP1 activity and expression is supported by studies that demonstrate enhanced thermogenic capacity to cold stress in repeated immobilization animals and an inability maintain body temperature with same cold stress in immobilization naïve animals [6].

Immobilization results in increased BAT thermogenic activity, increased energy metabolism [6, 8], and increased UCP1 function. In the present study, repeated immobilization resulted in reduced body

weight gain and increased energy dissipation as heat without increased energy intake.

Effects of immobilization on UCP1 function. Regulation of UCP1 thermogenic function is mediated by tonic release of NE from the sympathetic nerve terminals that innervate BAT [4]. Immobilization increases sympathetic activity in BAT, as estimated by NE turnover rate [32]. In the present study, acute immobilization increased UCP1 function irrespective of repeated immobilization exposure. The increased UCP1 function occurred without a concomitant increase in gene expression, suggesting that preexisting UCP1 was activated with immobilization. The increase of UCP1 activity from basal levels in response to an acute immobilization was significantly larger in repeatedly immobilized group than in the naive group. This may be partially explained by increased UCP1 expression in repeatedly immobilized group, yielding a higher absolute UCP1 activity when stimulated by acute immobilization.

We previously demonstrated that the BAT NE turnover rate was significantly higher in repeatedly immobilized rats than in naive rats when both groups were exposed to acute immobilization [32]. Glucocorticoids possess a powerful inhibitory effect on UCP1 function by attenuating SNS activity [33–35] and by directly suppressing UCP1 gene expression [17, 20]. In the present experiment, immobilization resulted in increased serum corticosterone level. While sham operated rats showed no change in UCP1 gene expression, ADX rats had an increased basal level of UCP1 mRNA, which increased even further in response to acute immobilization. Though an influence of interception of the HPA-axis in ADX experiments should be considered, many lines of evidence showing that glucocorticoids among several corticosterone inhibits UCP1 gene expression have been accumulated [17–20, 36, 37]. Taking these into account, our results suggest that increased level of circulating glucocorticoids during acute immobilization might override the facilitative effect of sympathetic stimulation on UCP1 mRNA expression. However, sustained elevation of circulating glucocorticoids, induced by repeated stress or by exogenous administration, downregulates cytosolic glucocorticoids receptors in the brain [35, 38, 39] and increases SNS activity and responsivity. This decreased sensitivity to glucocorticoids might be the one of mechanism by which UCP1 expression is increased in repeatedly immobilized rats.

The stability of the UCP1 mRNA is determined by its half-life (after transcriptional inhibition) and can change dramatically depending on the nature and duration of stimulation [40, 41]. When rats are exposed to acute cold stimulus, the half-life of UCP1 mRNA is reduced and may account for the rapid decrease in UCP1 gene expression after cessation of stimulation [42] while the prolongation of the half-life of UCP1 (70 h) after chronic adrenergic stimulation has also been observed [41]. Therefore, the elevated mRNA level 24 h after last immobilization in the repeated immobilization group may be dependent on neuroendocrine regulatory factors as well as changes in UCP1 mRNA stability itself.

Modification of body temperature regulation in immobilized rats. Acute immobilization induces hyperthermia in rats [43–45], which can be inhibited by BAT sympathectomy [46]. In the present experiment, core temperature increased after 3 h of acute immobilization, suggesting that BAT UCP1 activation may be directly linked to an increase of body temperature. The acute immobilization-induced increased in UCP1 activity were higher in the repeatedly immobilized rats than in naive group, suggesting that the increase in body temperature would be greater in the repeated immobilized group than in the naive group. However, there was no difference in core temperatures between these two groups following acute immobilization. This may be due to enhanced heat dissipation in repeatedly immobilized animals, as described previously [47].

Several studies demonstrated a stimulatory effect of ADX on BAT thermogenic capacity [17, 18, 48]. However, the present data showed that core temperature did not change in ADX animals in response to acute immobilization, despite an increase in both UCP1 activity and expression (Figs. 4 and 5). This may reflect a loss of glucocorticoid action in thermoregulation (i.e., preventing of heat preservation by vasoconstriction and prevention of mobilization of free fatty acids and glucose as fuel for thermogenesis [3]).

In the present experiments, we demonstrated that UCP1 function was enhanced by immobilization in rats. In particular, repeated immobilization resulted in upregulation of UCP1 expression and activity and in increased BAT NST capacity. This resulted in an enhanced ability to dissipate energy, maintain body temperature during cold exposure, and maintain lower body weight.

We thank Dr. P. G. Osborne (guest professor, Asahikawa Medical University) for his critical review of this manuscript. This work was partially supported by a Grant-in-Aid for Scientific Research (B) #09470014 to AK and HO and JSPS Grant-in-Aid for Encouragement of Young Scientists #12770029 to KK-U and Grant-in-Aid for Scientific Research (B) #14370018 to MH.

REFERENCES

1. Chrousos GP and Gold PW: The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267: 1244–1252, 1992
2. Johnson EO, Kamilaris TC, Chrousos GP, and Gold PW: Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. Neurosci Biobehav Rev 16: 115–130, 1992
3. Deavers DR and Musacchia XJ: The function of glucocorticoids in thermogenesis. Fed Proc 38: 2177–2181, 1979
4. Lowell BB and Spiegelman BM: Towards a molecular understanding of adaptive thermogenesis. Nature 404: 652–660, 2000
5. Pacak K, Palkovits M, Kvetnansky R, Yadid G, Kopin IJ, and Goldstein DS: Effects of various stressors on *in vivo* norepinephrine release in the hypothalamic paraventricular nucleus and on the pituitary- adrenocortical axis. Ann N Y Acad Sci 771: 115–1130, 1995
6. Kuroshima A, Habara Y, Uehara A, Murazumi K, Yahata T, and Ohno T: Cross adaption between stress and cold in rats. Pflügers Arch 402: 402–408, 1984
7. Lowell BB, Hamann SSVA, Lawitts JA, Himms-Hagen J, Boyer BB, Kozak LP, and Flier JS: Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. Nature 366: 740–742, 1993
8. Nozu T, Okano S, Kikuchi K, Yahata T, and Kuroshima A: Effect of immobilization stress on *in vitro* and *in vivo* thermogenesis of brown adipose tissue. Jpn J Physiol 42: 299–308, 1992
9. Himms-Hagen J: Brown adipose tissue thermogenesis: interdisciplinary studies. Faseb J 4: 2890–2898, 1990
10. Klingenberg M and Huang SG: Structure and function of the uncoupling protein from brown adipose tissue. Biochim Biophys Acta 1415: 271–296, 1999
11. Nicholls DG: The thermogenic mechanism of brown adipose tissue. Review. Biosci Rep 3: 431–441, 1983
12. Ricquier D, Casteilla L, and Bouillaud F: Molecular studies of the uncoupling protein. FASEB J 5: 2237–2242, 1991
13. Silva JE and Rabelo R: Regulation of the uncoupling protein gene expression. Eur J Endocrinol 136: 251–264, 1997
14. Kopecky J, Jezek P, Drahota Z, and Houstek J: Control of uncoupling protein in brown-fat mitochondria by purine nucleotides. Chemical modification by diazobenzenesulfonate. Eur J Biochem 164: 687–694, 1987
15. Tsigos C and Chrousos GP: Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53: 865–871, 2002
16. Casteilla L, Nougues J, Reyne Y, and Ricquier D: Differentiation of ovine brown adipocyte precursor cells in a chemically defined serum-free medium. Importance of glucocorticoids and age of animals. Eur J Biochem 198: 195–199, 1991
17. Moriscot A, Rabelo R, and Bianco AC: Corticosterone inhibits uncoupling protein gene expression in brown adipose tissue. Am J Physiol 265: E81–E87, 1993
18. Strack AM, Bradbury MJ, and Dallman MF: Corticosterone decreases nonshivering thermogenesis and increases lipid storage in brown adipose tissue. Am J Physiol 268: R183–R191, 1995
19. Soumano K, Desbiens S, Rabelo R, Bakopanos E, Camirand A, and Silva JE: Glucocorticoids inhibit the transcriptional response of the uncoupling protein-1 gene to adrenergic stimulation in a brown adipose cell line. Mol Cell Endocrinol 165: 7–15, 2000
20. Viengchareun S, Penfornis P, Zennaro MC, and Lombes M: Mineralocorticoid and glucocorticoid receptors inhibit UCP expression and function in brown adipocytes. Am J Physiol Endocrinol Metab 280: E640–E649, 2001
21. Akana SF, Dallman MF, Bradbury MJ, Scribner KA, Strack AM, and Walker CD: Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. Endocrinology 131: 57–68, 1992
22. Akana SF, Scribner KA, Bradbury MJ, Strack AM, Walker CD, and Dallman MF: Feedback sensitivity of the rat hypothalamo–pituitary–adrenal axis and its capacity to adjust to exogenous corticosterone. Endocrinology 131: 585–594, 1992.
23. Szymusiak R and Satinoff E: Maximal REM sleep time defines a narrower thermoneutral zone than does minimal metabolic rate. Physiol Behav 26: 687–690, 1981
24. Butterfield WC and Rasche R: An improved inexpensive method of restraint for the formation of stress ulcers in the rat. Rev Surg 32: 75–76, 1975
25. Cannon B and Lindberg O: Mitochondria from brown adipose tissue: isolation and properties. Methods Enzymol 55: 65–78, 1979
26. Nedergaard J and Cannon B: Apparent unmasking of [^{3}H]GDP binding in rat brown-fat mitochondria is due to mitochondrial swelling. Eur J Biochem 164: 681–686, 1987
27. Harris RB, Mitchell TD, Simpson J, Redmann SM Jr, Youngblood BD, and Ryan DH: Weight loss in rats exposed to repeated acute restraint stress is independent of energy or leptin status. Am J Physiol 282: R77–R88, 2002
28. Langer P, Foldes O, Kvetnansky R, Culman J, Torda T, and El Daher F: Pituitary-thyroid function during acute immobilization stress in rats. Exp Clin Endocrinol 82: 51–60, 1983
29. Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, and Cannon B: UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta 1504: 82–106, 2001
30. Nedergaard J, Matthias A, Golozoubova V, Jacobsson A, and Cannon B: 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, 1999
31. Trayhurn P and Milner RE: A commentary on the interpretation of in vitro biochemical measures of brown adipose tissue thermogenesis. Can J Physiol Pharmacol 67: 811–819, 1989
32. Murazumi K, Yahata T, and Kuroshima A: Effects of cold and immobilization stress on noradrenaline

turnover in brown adipose tissue of rat. Jpn J Physiol 37: 601–607, 1987
33. Brown MR and Fisher LA: Glucocorticoid suppression of the sympathetic nervous system and adrenal medulla. Life Sci 39: 1003–1012, 1986
34. Kvetnansky R, Fukuhara K, Pacak K, Cizza G, Goldstein DS, and Kopin IJ: Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats. Endocrinology 133: 1411-1419, 1993
35. Kvetnansky R, Pacak K, Fukuhara K, Viskupic E, Hiremagalur B, Nankova B, Goldstein DS, Sabban EL, and Kopin IJ: Sympathoadrenal system in stress. Interaction with the hypothalamic–pituitary–adrenocortical system. Ann NY Acad Sci 771: 131–158, 1995
36. Arvaniti K, Ricquier D, Champigny O, and Richard D: Leptin and corticosterone have opposite effects on food intake and the expression of UCP1 mRNA in brown adipose tissue of lep(ob)/lep(ob) mice. Endocrinology 139: 4000–4003, 1998
37. Bell ME, Bhatnagar S, Liang J, Soriano L, Nagy TR, and Dallman MF: Voluntary sucrose ingestion, like corticosterone replacement, prevents the metabolic deficits of adrenalectomy. J Neuroendocrinol 12: 461–470, 2000
38. Sapolsky RM, Krey LC, and McEwen BS: Stress down-regulates corticosterone receptors in a site-specific manner in the brain. Endocrinology 114: 287–292, 1984
39. Sapolsky RM and McEwen BS: Down-regulation of neural corticosterone receptors by corticosterone and dexamethasone. Brain Res 339: 161–165, 1985
40. Pico C, Herron D, Palou A, Jacobsson A, Cannon B, and Nedergaard J: Stabilization of the mRNA for the uncoupling protein thermogenin by transcriptional/translational blockade and by noradrenaline in brown adipocytes differentiated in culture: a degradation factor induced by cessation of stimulation? Biochem J 302: 81–86, 1994
41. Puigserver P, Herron D, Gianotti M, Palou A, Cannon B, and Nedergaard J: 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: 393–398, 1992
42. Jacobsson A, Cannon B, and Nedergaard J: Physiological activation of brown adipose tissue destabilizes thermogenin mRNA. FEBS Lett 224: 353–256, 1987
43. Beotra A and Sanyal AK: Immobilization stress induced hyperthermia in rats: possible role of noradrenaline. Indian J Exp Biol 20: 759–760, 1982
44. Kuroshima A and Yahata T: Changes in the colonic temperature and metabolism during immobilization stress in repetitively immobilized or cold-acclimated rats. Jpn J Physiol 35: 591–597, 1985
45. Shibata H and Nagasaka T: Contribution of nonshivering thermogenesis to stress-induced hyperthermia in rats. Jpn J Physiol 32: 991–995, 1982
46. Shibata H and Nagasaka T: Role of sympathetic nervous system in immobilization- and cold-induced brown adipose tissue thermogenesis in rats. Jpn J Physiol 34: 103–111, 1984
47. Kuroshima A, Yahata T, and Murazumi K: Thermal and metabolic responses of repetitively immobilized rats to acute heat exposure. Hokkaido Igaku Zasshi 61: 463–465, 1986
48. Marchington D, Rothwell NJ, Stock MJ, and York DA: Thermogenesis and sympathetic activity in BAT of overfed rats after adrenalectomy. Am J Physiol 250: E362–E366, 1986