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Original Contribution

THE CONSEQUENCES OF ACUTE COLD EXPOSURE ON PROTEIN OXIDATION AND PROTEASOME ACTIVITY IN SHORT-TAILED FIELD VOLES, *MICROTUS AGRESTIS*

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Abstract—During cold exposure, animals upregulate their metabolism and food intake, potentially exposing them to elevated reactive oxygen species (ROS) production and oxidative damage. We investigated whether acute cold (7 ± 3°C) exposure (1, 10, or 100 h duration) affected protein oxidation and proteasome activity, when compared to warm controls (22 ± 3°C), in a small mammal model, the short-tailed field vole *Microtus agrestis*. Protein carbonyls and the chymotrypsin-like proteasome activity were measured in plasma, heart, liver, kidney, small intestine (duodenum), skeletal muscle (gastrocnemius), and brown adipose tissue (BAT). Trypsin-like and peptidyl-glutamyl-like proteasome activities were determined in BAT, liver, and skeletal muscle. Resting metabolic rate increased significantly with duration of cold exposure. In skeletal muscle (SM) and liver, protein carbonyl levels also increased with duration of cold exposure, but this pattern was not repeated in BAT where protein carbonyls were not significantly elevated. Chymotrpsin-like proteasome activity did not differ significantly in any tissue. However, trypsin-like activity in SM and peptidyl-glutamyl-like activity in both skeletal muscle and liver, were reduced during the early phase of cold exposure (1–10 h), correlated with the increased carbonyl levels in these tissues. In contrast there was no reduction in proteasome activity in BAT during the early phase of cold exposure and peptidyl-glutamyl-like activity was significantly increased, correlated with the lack of accumulation of protein carbonyls in this tissue. The upregulation of proteasome activity in BAT may protect this tissue from accumulated oxidative damage to proteins. This protection may be a very important factor in sustaining uncoupled respiration, which underpins nonshivering thermogenesis at cold temperatures. © 2002 Elsevier Science Inc.

Keywords—Protein oxidation, Proteasome, Resting metabolic rate, Cold exposure, Free radicals

INTRODUCTION

Reactive oxygen species (ROS) are produced both as a byproduct of cellular metabolism in aerobic animals and also from various environmental sources [1,2]. When ROS production exceeds the capacities of protection and repair mechanisms oxidative stress occurs, resulting in damage to macromolecules such as proteins, lipids, and DNA [2]. The oxidation of proteins can lead to decline in both protein function and enzyme activity [2–5]. The relative risk of oxidative stress appears to increase during periods of elevated metabolism, including both exercise activity and cold exposure [6–8].

In mammalian cells, the breakdown of oxidized proteins occurs via two distinct pathways, the lysosomal and proteasomal systems [4,5,9–11]. The proteasome is a multicatalytic cytosolic and nuclear proteinase complex [12], which appears present in all eukaryotic cells, mitochondria, and bacteria [4]. The most widely studied function of the proteasome has been its role in the selective and rapid degradation of oxidized proteins. This rapid degradation, both in the presence and absence of ATP and ubiquitin, appears necessary to reduce the potential for aberrant protein accumulation. Indeed, an in-

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crease in various oxidized proteins appears indicative of the aging process, and may result from an increase in oxidative stress, a decrease in proteasome activity, or a combination of both [3,4,12,13]. Oxidative stress itself may be a causative factor in proteasome inhibition, leading to a vicious cycle, which results in the progressive increase in protein oxidation (e.g., [12]). Indeed, proteasome inhibition has been cited as a causative factor in certain neurodegenerative disorders such as Alzheimer's disease [11].

In small mammals that do not enter hibernation or torpor, survival at low temperatures is subject to the individual's ability to increase heat production to maintain core body temperature. This is achieved through shivering and nonshivering thermogenesis (NST) [14]. Shivering thermogenesis occurs in skeletal muscle and is indicative of acute cold exposure. The decline in thermogenesis through shivering thermogenesis over time following chronic cold exposure is matched by an increase in NST, thereby maintaining body temperature [14,15]. The main site of NST is brown adipose tissue (BAT) [16–19]. NST in BAT is achieved by the uncoupling of oxidative metabolism from ATP production by uncoupling protein-1 (UCP-1) in mitochondria, resulting in the generation of heat [20,21]. In small mammals, UCP-1 expression increases within hours of cold exposure [22]. BAT may account for up to 60% of total oxygen consumption, but only 1% of total body mass during cold exposure [15].

The levels of several endogenous antioxidants [6,23, 24], antioxidant enzymes [23,25–27], and heat shock proteins [28] have been shown to increase significantly in several tissues, including BAT, skeletal muscle, and liver after both acute and chronic cold exposure. The generation of hydrogen peroxide in BAT increased by over 2-fold and superoxide radical ($O_2^{\bullet-}$) production was higher in rats after 30 d cold exposure [17], compared to those levels prior to the cold exposure. Indeed, it has been calculated that, with the accompanying increase in mitochondria number, cold exposure in rats resulted in a 6–9-fold increase in $O_2^{\bullet-}$ generation per mg of BAT [17]. Consequently it has been suggested that during cold exposure, BAT is highly susceptible to oxidative stress, primarily due to its high mass-specific metabolic rate, high mitochondrial concentration, and the increased generation of ROS [17]. Additional tissues such as liver and skeletal muscle may also be prone to cold-induced oxidative stress due to hypertrophy [30], an increased metabolic rate, changes in capacity for β-oxidation and in the oxidative capacity of mitochondria [31].

To sustain core body temperature, an increase in metabolism is required immediately on exposure to low ambient temperatures. This elevation in metabolism may increase the production of ROS, possibly before any induction of the antioxidant protection and repair mechanisms takes place. Indeed, the activity of superoxide dismuthase in BAT was reduced in rats after acute (6 h) cold exposure compared to the levels of control and chronic (21 d) cold-exposed animals [27]. Therefore, it is likely that during acute cold exposure, the disparity between ROS production and the protection and repair mechanisms should be at its greatest, which may result in a significant risk of oxidative stress at this time.

The aims of the present study were to examine whether acute cold (7°C) exposure (1, 10, or 100 h duration), altered the chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-like peptidase activities of the proteasome complex in various tissues when compared to warm controls (22°C), and whether differences occurred, within the same tissue, in these activities. Protein carbonyl levels were also measured in various tissues of a small mammalian model, the short-tailed field vole *Microtus agrestis*, including those involved in shivering thermogensis and NST, i.e., BAT and skeletal muscle [29] and in those involved in the increase in food intake, i.e., small intestine and liver [30]. We measured resting metabolic rate in a sub-sample of individuals from each experimental group. Previously we showed that long-term cold exposure increased metabolic rate, body mass, food intake rate [30], and catalase and glutathione peroxidase activities in various tissues [26] in these animals and that they remain active and maintain body temperature at low temperature, exhibiting neither torpor nor hibernation [32].

MATERIALS AND METHODS

Animals

Short-tailed field voles *Microtus agrestis* were bred from a captive population housed in Aberdeen, UK. All individuals were born at 22 ± 3°C, which is slightly below the thermoneutral zone (25–30°C) of these small (15–30 g) microtine rodents. Voles were weaned aged 18 d and housed individually in cages (48 × 15 × 13 cm) with sawdust and ad libitum access to water and a pelleted rodent diet (rat and mouse breeder and grower diet, Special Diets Services, BP Nutrition, Essex, UK), which included vitamin E (103.2 mg/kg), β-carotene (0.9 mg/kg), and vitamin C (8.0 mg/kg). Photoperiod was maintained at 16L: 8D, with lights on at 0500 h GMT. All voles were sacrificed humanely, complying with a local ethical committee and under license from the U.K. Home Office.

Cold exposure protocol

Same-sex sibling quadruplets (4 male and 4 female) were used during this study to reduce genetic variability

within experimental groups. At ≅50 d of age, one individual from each sibling group remained at 22 ± 3°C, while each of the other 3 were exposed to an ambient temperature of 7 ± 3°C in a controlled temperature room for 1, 10, or 100 h. All individuals had access to ad libitum food and water during these periods but did not have access to bedding material. To reduce any diurnal effects, all individuals were weighed (Sartorius, Essex, UK, 0.01 g) and subsequently sacrificed between 1200 and 1300 h GMT. Immediately after death, various tissues (heart, liver, skeletal muscle, kidney, brown adipose tissue, duodenum) were dissected out within 60 s of death, snap frozen in liquid N_2 and stored at −80°C until required. Blood samples were also collected, spun at 3000 rpm and the resulting supernatant collected, snap frozen in liquid nitrogen, and stored at −80°C.

Resting metabolic rate

Measurements of resting metabolic rate were collected on an age-matched group of 16 animals (8 male and 8 female). Resting metabolic rate measurements were collected following exactly the protocol and time of death (1200–1300 h GMT) of the acute cold exposure experiment, i.e., control (22°C), 1, 10, and 100 h cold exposure (7°C) with 2 males and 2 females in each group. Those individuals that were cold exposed for either 10 or 100 h were transferred to the cold rooms (7 ± 3°C), as before, and then transferred to a controlled-temperature incubator (INL-401N-010, Gallencamp, Loughborough, UK) set to 7°C, at 0900 h GMT. Individuals cold exposed for 1 h were transferred to the incubators, set at 22°C at 0900 h GMT, and then the temperature switched to 7°C at 1130 h GMT and warm control individuals were transferred to the incubators, set at 22°C, at 0900 h GMT.

Resting metabolic rate was quantified as the rate of oxygen consumption, during the light phase, using an open-flow respirometry system described fully elsewhere [33]. In brief, voles were placed individually within a sealed Perspex chamber contained in a controlled-temperature incubator. Air, dried through silica gel (BDH, Middlesex, UK) was pulled through the system (Charles Austin Pump Ltd, Leighton Buzzard, UK), at a rate of 600–800 ml min^{-1} (DM3A, Alexander-Wright flow meter, London, UK). Air was then re-dried and a sample (150 ml min^{-1}) was passed to the oxygen analyzer (Series 1100, Servomex plc, Crowburgh, UK). To maximize accuracy of our resting metabolic rate measurements, carbon dioxide was not absorbed before measuring oxygen consumption [34]. The recordings were collected over 30 s periods and directly downloaded to a microcomputer and the lowest 10 readings (equivalent to 5 min^{-1}) during 1200 and 1300 h GMT were used to estimate resting metabolic rate. All samples were subsequently corrected for pressure and temperature [35].

Proteasomal activity

The degradations of the fluoropeptide suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like), suc-Leu-Leu-Glu-AMC (trypsin-like), and suc-Pro-Phe-Arg-AMC (peptidyl-glutamyl-like) were measured after addition of the substrate to the tissue. Protein was determined using a Bio-Rad kit. Twenty microliters of tissue homogenates were incubated with 170 μl proteolysis buffer (0.15 M sucrose, 25 mM Hepes (pH 7.8), 20 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol) and with 10 μl of the indicated fluropeptide (2 mM stock solution in DMSO). The mixture was incubated for 30 min at 37°C, and the reaction then stopped by addition of an equal volume of ice-cold 96% ethanol. The fluorescence determination was performed at 380 nm excitation and 440 nm emission using free MCA as a standard.

Carbonyl measurement

Protein carbonyl content was determined on the homogenized tissue supernatant with a concentration of proteins between 0.5 and 5 mg/ml employing an ELISA method [36,37], with the appropriate modifications steps [38]. The detection system used was an anti-dinitrophenyl rabbit IgG-antiserum (Sigma, Deisenhofen, Germany) as the primary antibody and a monoclonal anti-rabbit IgG antibody peroxidase conjugate (Sigma) as the secondary antibody. Development was performed with o-phenylenediamine.

Statistics

All values reported are mean ± standard error (SEM), except where otherwise indicated. Data were analyzed employing SPSS (Version 9) and Minitab (Version 11) statistical software employing one-way and repeated-measures analysis of variance and linear regression analysis. Differences between experimental groups were examined using Tukey-tests. Significance was indicated where p values $< .05$.

RESULTS

Resting metabolic rate

Resting metabolic rate was significantly lower ($p < .001$) in the warm (22 ± 3°C) control group compared those individuals exposed to the cold (7 ± 3°C) for 1, 10, or 100 h (Fig. 1). No significant differences were observed between any of the cold-exposed groups. There

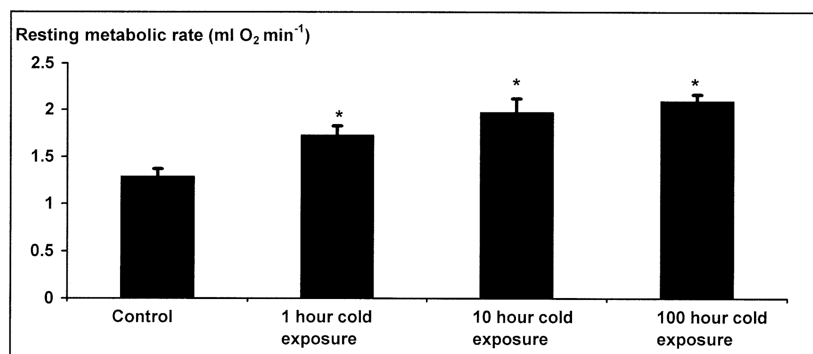

Fig. 1. Mean ± SEM resting metabolic rate measured in warm control, 1, 10, and 100 h cold-exposed voles ($n = 6$ in each group). RMR was significantly lower in controls when compared to all other groups ($p < .001$) and RMR significantly increased with time in the cold ($p = .001$). Significant differences to control values denoted by *.

was also a significant time effect with resting metabolic rate increasing with time in the cold ($p < .000$). Resting metabolic rate was 35% higher after 1 h in the cold, 53% higher after 10 h in the cold, and 63% higher after 100 h in the cold compared to warm control levels. Body mass (mean ± SEM) in warm controls (20.7 ± 1.1 g), 1 h (21.0 ± 0.8 g), 10 h (21.0 ± 1.1 g) and 100 h (20.8 ± 0.7 g) cold exposed were not significantly different from one another ($p = .996$).

Protein carbonyl levels

The levels of protein carbonyls were significantly different between cold exposure groups in both liver ($p = .025$) and small intestine ($p = .013$). A significant time effect was also observed in these tissues, with carbonyl levels increasing significantly with time exposed to the cold in both the liver (Fig. 2; $p = .041$) and small intestine (Fig. 2; $p < .001$). No other tissues showed any significant group or time effects in protein carbonyl levels. However, a significant difference in protein carbonyl levels were seen between tissues ($p < .000$), with brown adipose tissue (BAT) having significantly higher levels than all other tissues and plasma having significantly elevated levels compared to all other tissues except BAT.

Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like) proteasome activity

No significant differences in chymotrypsin-like proteasome activity were observed between experimental groups in liver, kidney, heart, BAT, skeletal muscle, plasma, or small intestine and no significant time effects seen between groups (Table 1).

Suc-Leu-Leu-Glu-AMC (trypsin-like) proteasome activity

Experimental groups did not differ in the trypsin-like proteasome activity (Table 2) in either the liver ($p = .540$) or BAT ($p = .492$). However, the activity in skeletal muscle did significantly differ between groups ($p = .029$), with the lowest measured at 1 and 10 h cold

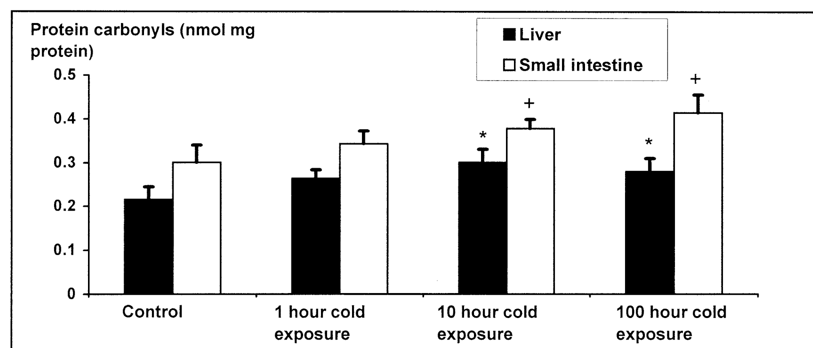

Fig. 2. Mean ± SEM liver and small intestine protein carbonyl levels. There was a significant group ($p = .025$; $p = .013$) and time effect ($p = .041$; $p < .001$) for liver and small intestine respectively. ($n = 8$ in each group). Significant differences to control levels denoted by * in the liver and by + in the small intestine.

Table 1. Mean ± SEM Activity of Chemotrypsin-like Proteasome Activity in Experimental Voles

	Control	1 h cold exposure	10 h cold exposure	100 h cold exposure	ANOVA	Time effect
Liver	8.97 ± 1.2	8.59 ± 0.91	7.66 ± 0.95	8.29 ± 0.96	p = .244	p = .222
Kidney	4.40 ± 0.13	4.11 ± 0.15	3.90 ± 0.30	4.40 ± 0.42	p = .321	p = .884
Heart	2.63 ± 0.26	2.54 ± 0.38	2.55 ± 0.27	2.26 ± 0.33	p = .618	p = .212
BAT	8.52 ± 0.73	7.44 ± 0.68	7.80 ± 0.95	8.90 ± 0.70	p = .351	p = .467
Skeletal muscle	0.93 ± 0.20	0.90 ± 0.13	0.95 ± 0.25	1.16 ± 0.18	p = .281	p = .070
Small intestine	224.3 ± 11.5	208.1 ± 15.8	209.4 ± 11.1	212.0 ± 11.9	p = .343	p = .424

Repeated measure analysis of variance (ANOVA) and linear regression analysis (time effects), where p = statistical significance. BAT = Brown adipose tissue.

exposure compared to control and 100 h cold exposure (Table 2).

Suc-Pro-Phe-Arg-AMC (peptidyl-glutamyl peptidase-like) proteasome activity

Significant differences between experimental groups in the activity of the peptidylglutamyl peptidase-like proteasome activity were observed in liver (p = .008), skeletal muscle (p = .049), and BAT (p = .001), with the activity in BAT significantly increasing with time in the cold (Table 2, p = .009). This time effect is primarily due to the 50% increase in activity between warm controls and individuals exposed to 100 h cold exposure. In both liver and skeletal muscle, however, the lowest levels were observed after 1 h cold exposure.

DISCUSSION

This study was undertaken primarily to examine the effect of acute cold (7 ± 3°C) exposure of 1, 10, and 100 h duration on protein oxidation and proteasome activity in various tissues of a small mammalian model, the short-tailed field vole. To maintain core body temperature below their lower critical temperature, the voles in this study elevated their metabolic rate significantly in a time-dependent manner in the cold. Cold-induced increases in metabolism in mammals are achieved initially by shivering thermogenesis in skeletal muscle [14,16] and subsequently by nonshivering thermogenesis (NST), primarily in brown adipose tissue (BAT) [17–19].

Protein carbonyl levels differed significantly between experimental groups only in the liver and small intestine, which exhibited increases in carbonyl levels in a time-dependent manner. Typically, mammals exposed to low temperatures respond initially by increasing food intake. This is followed later with hypertrophy of the liver and small intestine. Cold exposure does appear to increase the metabolic rate of these tissues [31], possibly as a response to the assimilation and processing of the increased food intake at this time. However, we suggest these tissues also appear to be under increased oxidative stress due to the elevated levels of protein carbonyls observed.

Skeletal muscle also significantly increases in metabolic rate during acute cold exposure, due primarily to shivering activity (although skeletal muscle may also play a minor role in NST [18,19]). However, there was no evidence of an increased rate of protein oxidation in skeletal muscle in this study. It has previously been shown that cold exposure of only 6 h duration was sufficient to upregulate the activities of CuZn SOD, Mn SOD, and catalase in rat skeletal muscle [27]. Therefore, the upregulation of antioxidant protection mechanisms may be sufficient in skeletal muscle to prevent significant protein oxidation, despite the increased metabolic rate. It is well established that amino acids released from muscle tissue are used for energy supply [39–41]. Since the

Table 2. Mean ± SEM Trypsin-like (T-L) and Peptidyl-glutamyl-like (P-G-L) Proteasome Activity in Experimental Groups

	Control	1 h cold exposure	10 h cold exposure	100 h cold exposure	ANOVA	Time effect
Liver (T-L)	43.35 (2.86)	40.36 (3.48)	42.75 (3.29)	40.66 (4.32)	p = .540	p = .544
Muscle (T-L)	6.13 (0.90)	5.49 (0.70)	5.35 (0.80)	6.08 (0.74)	p = .029	p = .748
BAT (T-L)	44.13 (4.76)	38.77 (4.51)	37.23 (4.74)	40.60 (4.10)	p = .492	p = .704
Liver (P-G-L)	10.48 (0.53)	9.41 (0.53)	9.71 (0.51)	9.90 (0.52)	p = .008	p = .492
Muscle (P-G-L)	1.39 (0.22)	1.29 (0.22)	1.27 (0.21)	1.42 (0.25)	p = .049	p = .375
BAT (P-G-L)	1.00 (0.12)	0.97 (0.13)	0.95 (0.11)	1.52 (0.19)	p = .001	p = .009

Repeated measure analysis of variance (ANOVA) and linear regression analysis, where p = statistical significance.

proteasome is involved in protein breakdown, we are unable to rule out the possibility that some proteins were degraded to increase the energy supply at this time. On the other hand, the animals used in our experiments were not starving and were able to increase their food intake, therefore enhanced food intake seems to be more likely to balance the energy amount in comparison to endogenous protein breakdown. BAT exhibited significantly higher protein carbonyl levels than all other tissues. This may be in part because protein carbonyls have their origin, in part, in oxidized lipids [5]. In rats after 6 h cold exposure, the activities of both CuZn SOD and MnSOD were reduced in BAT compared to warm controls. Therefore, unlike in skeletal muscle (see [27]), antioxidant enzyme induction in BAT appears to occur over a longer time scale.

Although there were no significant changes in the chymotrypsin-like proteasome activity with cold exposure in any tissue, there were decreases in trypsin-like activity in skeletal muscle and peptidyl-glutamyl-like activity in liver and skeletal muscle initially on exposure to the cold, which may be due to proteasome regulation. The proteasome is regulated by numerous factors, and an intracellular modification of proteasome activity may take place during this short time period in the cold. Additionally, due to the elevated oxidative stress in the cold, more oxidized proteins are formed and therefore the catabolic flux rate via the proteasome will increase. It is known that some oxidatively modified proteins, e.g., β-amyloid aggregates, are able to inhibit the proteasome [12] and that their accumulation is toxic and their removal is critical for cell function. These downregulatory phenomena may result in decreased activity in the short term (1–10 h), which is ultimately overwhelmed during the acclimation process of cold exposure. This may be why proteasome activity approaches, or exceeds that of warm control levels, after 100 h cold exposure. The proteasomal system appears to be a highly regulated system, with differences in regulation occurring in various tissues in response to acute cold exposure. We propose that proteasomal inactivation after 1 h cold exposure in liver and skeletal muscle may be, at least in part, due to the effects of oxidative stress at this time. Our data indicate that the different proteasomal activities, e.g., chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-hydrolyzing activities, within the same tissue, react differently to acute cold exposure, with the greatest differences seen in the trypsin-like and the peptidyl-glutamyl-hydrolyzing activities of the proteasome. This is in agreement with other data previously published by our group [42,43] and fellow scientists [44,45].

In BAT, however, there was no evidence of a significant reduction in any proteasome activity, with the peptidyl-glutamyl-like activity actually significantly increasing with time in the cold. BAT is the principle thermogenic organ during cold exposure in small mammals [16,18,19]. This absence of a reduction in proteasome activity in BAT during 1–10 h cold exposure, compared with the liver and skeletal muscle, may enable BAT to counteract the potentially high oxidative stress encountered during acute cold exposure [6,17]. This elevation of proteasome activity correlated with the fact that no significant increase in protein oxidation occurred in BAT during cold exposure. It is not known how protein oxidation affects the thermogenic ability of BAT, although there is evidence that modification of proteins by oxygen radicals results in a reduction in enzymatic activity and/or protein function [4,5]. Any such reduction in the thermogenic capacity of BAT would likely have severe implications for the survival of a small mammal during cold exposure. This may be a factor explaining why there was no apparent decrease in proteasome activity in the cold. The mechanism by which BAT avoids the downregulation of proteasome activity observed in skeletal muscle and liver remains obscure, but a potentially important system which may provide future insights into regulation of the proteasome.

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ABBREVIATIONS

BAT—Brown adipose tissue
NST—Nonshivering thermogenesis
ROS—Reactive oxygen species
UCP 1—Uncoupling protein 1