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Leptin immunoexpression and innervation in rat interscapular brown adipose tissue of cold-acclimated rats: the effects of L-arginine and L-NAME

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Abstract: The aim of the present study was to explore the effect of nitric oxide on leptin immunoexpression and innervation in interscapular brown adipose tissue (IBAT) of room- and cold- acclimated rats. Animals acclimated both to room-temperature (22 ± 1°C) and cold (4 ± 1°C) were treated with L-arginine, a substrate for nitric oxide synthases (NOSs), or N?-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOSs, for 45 days. Leptin expression and localization in brown adipocytes was studied by immunohistochemistry, and innervation stained by the Bodian method. Strong leptin immunopositivity was observed in brown adipocytes cytoplasm of all room-acclimated groups, but nuclear leptin positivity was found only in L-NAME treated rats. In cold-acclimated control and L-NAME treated rats leptin immunopositivity was absent, while L-arginine treatment reversed the cold-induced suppression of leptin expression. Comparing to control, L-arginine, and even more L-NAME, at 22 ± 1°C induced greater innervation. In conclusion, L-arginine treatment changes leptin expression pattern on cold in rat IBAT.

Key words: IBAT - Leptin - Nitric oxide - Innervation - Cold

Introduction

Interscapular brown adipose tissue (IBAT) in small mammals serves as the main site of thermogenesis in a cold environment [1-4]. The key element in IBAT thermogenesis is the unique expression of a mitochondrial uncoupling protein-1 (UCP1). UCP1 is a proton carrier that, upon activation, causes uncoupling of respiration from oxidative phosphorylation, thus causing dissipation of energy as heat. The UCP1 gene is under strict transcriptional control associated with heat demand.

The essential role in the regulation of UCP1 gene expression plays the sympathetic nervous system (SNS) which abundantly innervates IBAT [2,5]. UCP1 gene induction in IBAT is mediated by sympathetic activation of β3-adrenergic receptors (β_3-AR) [6].

Considerable progress has recently been achieved in analyzing other mechanisms that control UCP1 expression. Scarpace *et al.* [7] showed that leptin administration causes an increase in UCP1 mRNA levels in IBAT and augments energy expenditure. However, this effect is also mediated through the SNS, because leptin is incapable to increases UCP1 mRNA in cultured brown adipocytes and denervated brown adipose tissue [8,9].

Leptin is a hormone that plays an important role in energy homeostasis [10,11]. Leptin is expressed and secreted both in white and brown adipose tissues [12] and acts as a signal for the level of adiposity. This hormone suppresses food intake and also increases energy expenditure [7,13].

It was shown that leptin increases core temperature and noradrenalin turnover in IBAT [11]. Namely, by stimulating sympathetic nerve activity [14] leptin increases the thermogenic activity in brown adipose tissue. It seems that food intake and IBAT thermogenesis are mutually regulated and signals for their regulation are peripherally and centrally integrated, although the key signal is still unknown.

Among the key signals found in practically all tissues, including the adipose tissue was nitric oxide (NO). It is one of biological messengers that can serve

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peripherally as well as in the central nervous system [15]. In addition, there is accumulating evidence for NO being involved in temperature regulation in homeotherms. In the periphery, modulation of thermoregulatory heat generation, especially in IBAT as the site of non-shivering thermogenesis, is discussed as NO action.

Brown adipocytes are capable to produce NO through two isoforms of nitric oxide synthase, inducible (iNOS) and endothelial (eNOS) [16,17]. In physiological conditions, its production is directly dependent on sympathetic activity. NO generation represents an important mechanism of modulating different IBAT functions, including vasodilatation of IBAT microcirculation [18], brown adipocytes proliferation [19], and mitochondriogenesis [20]. We also showed recently that the NO plays an important role in the regulation of IBAT function [21].

A few studies have investigated leptin localization [22,23] and expression [12,24] in white and brown adipocytes, but their relationship with sympathetic innervation is still ambiguous. To date no considerable data have yet appeared that deal with regulatory mechanisms involved in leptin expression in IBAT of cold-acclimated rats, with the focus on the effects of NO and innervation.

Given these considerations, we subjected the rats to a long term cold exposure (45 days, 4 ± 1°C), which is known to increase thermogenic capacity, and treated them with L-arginine (a substrate for NOSs) or N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOSs, in order to investigate potential effects of NO on leptin expression and innervation in IBAT.

Materials and Methods

Animals and experimental design. Mill Hill hybrid hooded, 4 months old rat *Rattus norvegicus* Berkenhout 1769 males were divided into three main groups. The first group received L-arginine HCl (2.25%), a substrate for NOSs, and the second group was given L-NAME HCl, (0.01%), an inhibitor of NOSs, in drinking water for 45 days. The doses of L-NAME and L-arginine used here did not induce any toxic effects, as confirmed otherwise [53]. The third group served as control. All three groups were additionally divided into two subgroups, one was kept at 22 ± 1 °C and the other in a cold room at 4 ± 1°C. The rats were maintained in individual cages, with food and water *ad libitum*. Each experimental group consisted of six animals. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*. The animals were sacrificed by decapitation. Immediately after removal, the samples of IBAT were fixed in a 10% formaldehyde solution at 4°C overnight and processed routinely for embedding in paraffin.

Immunohistochemistry. A series of 5 μm thick IBAT sections were deparaffinized and rehydrated. Immunoreactivity was assessed by the avidin-biotin-peroxidase method (Santa Cruz Biotechnology manual). The sections were incubated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature to block endogenous peroxidase, followed by three washes in 0.015 M phosphate-buffered saline (PBS, pH=7.4) of 5 minutes each, and incubation with a 1.5% normal goat serum (ABC Staining System, Santa Cruz Biotechnology Santa Cruz, CA, USA) in PBS for 60 minutes at room temperature to block non-specific binding. The primary antibody against leptin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was polyclonal antibodies produced in rabbit. The sections with primary antibodies were incubated overnight at 4°C, followed by two PBS washes of 5 minutes each; then incubated with 1:200 IgG biotinylated serum goat anti-rabbit (ABC Staining System, Santa Cruz Biotechnology) in PBS for 30 minutes at room temperature, followed by two PBS washes of 5 minutes each. AB reagent (ABC Staining System, Santa Cruz Biotechnology) was added for 60 minutes at room temperature, followed by three PBS washes of 5 minutes each, and then incubated with 0.02% H_2O_2 and 0.075% diaminobenzidine (Sigma-Aldrich, Inc. St Louis, MO, USA) in 0.05 M Tris buffer, pH=7.6, for 10 minutes in a dark room. The sections were rinsed in distilled water, counterstained with hematoxylin, and then observed with a Leica DMLB (Austria) microscope. Negative controls were prepared by omitting the primary antibody.

Bodian's nerve staining. IBAT sections of 5 μm thickness were used for Bodian's nerve staining [25].

Results

The changes in body mass during acclimation showed characteristic pattern: initially, in cold acclimation animals from all examined groups lost body mass which returned to control level at 45 day of cold-acclimation. During the whole experiment no changes in fluid intake were observed. In contrast, as acclimation to cold started, animals in all examined groups increased food intake by 100% and that increase remained constant to the end of experiment (data not shown).

Leptin immunohistochemistry

Brown adipocytes from rats acclimated to room temperature (22 ± 1°C) show strong cytoplasmic immunopositivity for leptin. Leptin expression was found both in unilocular and multilocular adipocytes types, although the majority of adipocytes were unilocular (Fig. 1A). In cold-acclimated rats all brown adipocytes were multilocular and leptin-negative (Fig. 1D).

L-arginine administration at 22 ± 1°C resulted in higher numbers of multilocular adipocytes compared to control, and leptin immunopositivity remained strong (Fig. 1B). L-arginine administration of cold-acclimated rats induced leptin expression. All adipocytes were leptin-positive (Fig. 1B), but the reaction was less intense than at 22 ± 1°C.

L-NAME treatment at 22 ± 1°C also potentiates multilocularity of brown adipocytes, even slightly more than L-arginine treatment (Fig. 1C). Leptin immunopositivity in brown adipocytes cytoplasm was found to be diminished, while numerous brown adipocyte nuclei had strong leptin positivity (Fig. 1C). In cold-acclimated rats treated with L-NAME, brown adipocytes were multilocular and no leptin-positive cells were found (Fig. 1F), similar to cold-acclimated, non treated rats (Fig. 1D).

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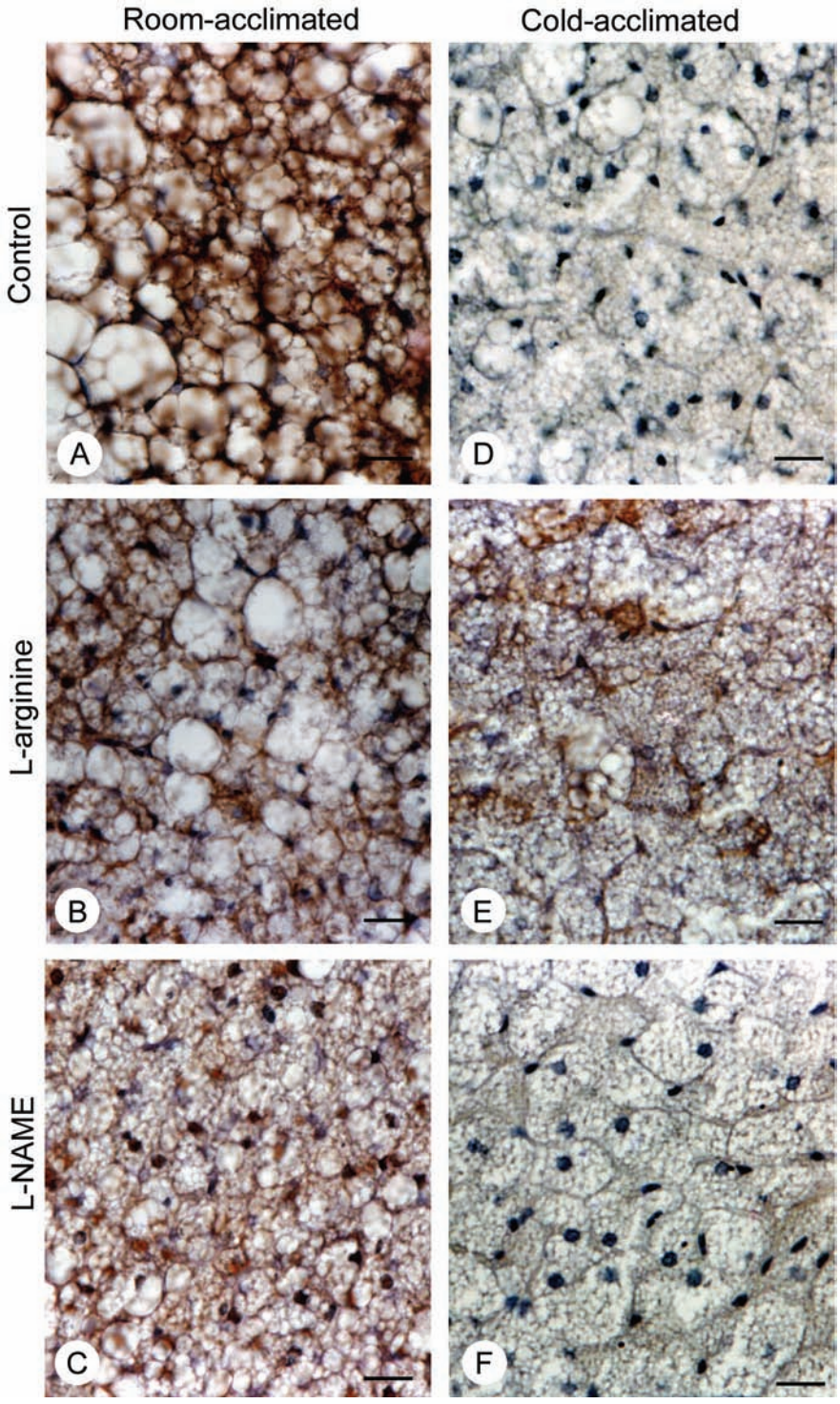

Fig. 1. Immunohistochemical detection of leptin in IBAT of room-acclimated (**A, B, C**) and cold-acclimated (**D, E, F**) rats. (**A**) Control 22 ± 1°C, (**D**) control 4 ± 1°C, (**B**) L-arginine 22 ± 1°C, (**E**) L-arginine 4 ± 1°C, (**C**) L-NAME 22 ± 1°C, (**F**) L-NAME 4 ± 1°C. At room temperature (22 ± 1°C) brown adipocytes from control and L-arginine treated rats show strong cytoplasmic immunopositivity for leptin (Fig. 1A, B), while in L-NAME treated rats leptin immunopositivity in brown adipocytes cytoplasm diminished, while numerous brown adipocyte nuclei have strong leptin positivity (Fig. 1C). In control and L-NAME treated, cold-acclimated rats all brown adipocytes are leptin-negative (Fig. 1D, F), while L-arginine administration induced leptin expression. All adipocytes were leptin-positive (Fig. 1E), but the reaction was less intense than at 22 ± 1°C (magnification ×40, original scale bars 50 μm).

Bodian's staining of innervation

Innervation in IBAT of control rats, acclimated to room temperature (22 ± 1°C) was slight (Fig. 2A). In L-arginine (Fig. 2C) and L-NAME (Fig. 2E) administered groups at 22 ± 1°C intensive innervation was found. All cold-acclimated groups had intensive innervation and no apparent differences between treatments were observed (Fig. 2B, D, F).

Discussion

In the present study we addressed leptin immunolocalization and innervation in IBAT of room- and cold-acclimated rats and a potential role of endogenously produced NO on their modulation. The major findings are the following.

Expression of leptin in IBAT was found to be correlated with ambient temperature: at room temperature

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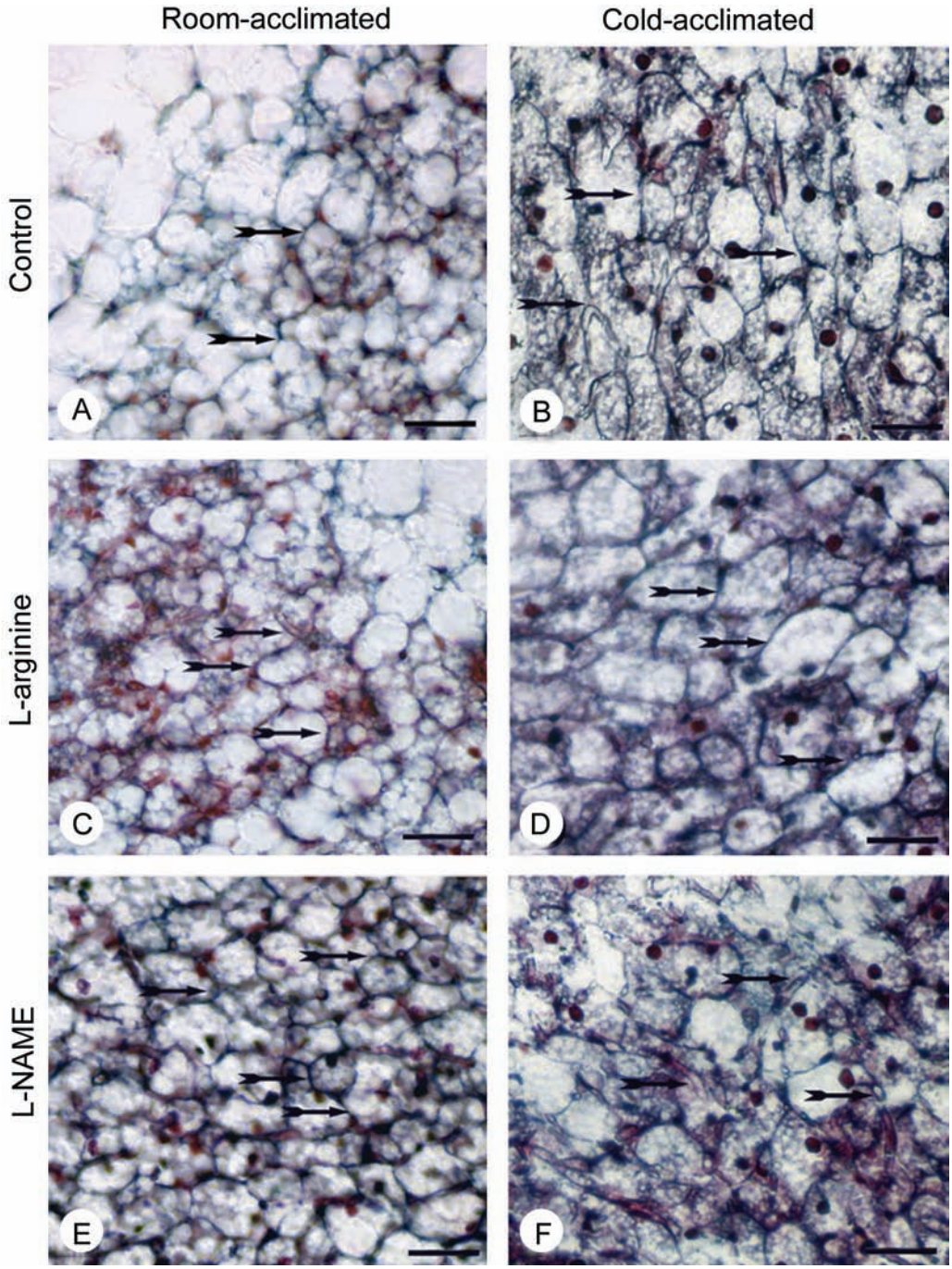

Fig. 2. Bodian's staining of innervation in IBAT of room-acclimated (**A, C, E**) and cold-acclimated (**B, D, F**) rats. (**A**) Control 22 ± 1°C, (**B**) control 4 ± 1°C, (**C**) L-arginine 22 ± 1°C, (**D**) L-arginine 4 ± 1°C, (**E**) L-NAME 22 ± 1°C, (**F**) L-NAME 4 ± 1°C. While in control rats, acclimated to room temperature (22 ± 1°C) (Fig. 2A) IBAT is slightly innervated, in L-arginine (Fig. 2C) and L-NAME (Fig. 2E) administered groups at 22 ± 1°C intensive innervation are found. All cold-acclimated groups have intensive innervation (Fig. 2B, D, F). Nerves - black arrows (magnification x40, original scale bars 40 μm).

(22 ± 1°C) leptin was strongly expressed, while it was absent in cold. In addition, leptin expression correlates well with the histological appearance of brown adipocytes and innervation. In room-acclimated rats, we observed unilocular appearance in the majority of brown adipocytes, weak innervation and leptin expression in brown adipocytes, closely related to thermogenic inactivity. Although some data concerning leptin expression are controversial, brown adipocytes in vivo and in vitro synthesize and release this hormone [26]. An open question is which type of brown adipocytes express leptin? Cinti *et al.* [22] have shown immunohistochemically that leptin is produced by the thermogenic inactive, unilocular brown adipocytes. Our

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results show that, unexpectedly, both types of brown adipocytes express leptin. It remains unclear, however, why multilocular brown adipocytes of room-acclimated rats express leptin.

Detailed mechanisms through which NO realizes this action in IBAT have still not been elucidated. Kikuchi-Utsumi *et al.* [27] have shown that cold exposure, which is known to stimulate noradrenaline release from sympathetic nerve terminals in IBAT, led to a significant increase in eNOS mRNA in this tissue. It is also known that eNOS is responsible for the physiological regulation of blood flow as well as for thermogenesis in IBAT and that eNOS activity and expression my be controlled by sympathetic nerve activity [27,28]. In contrast, very low levels of iNOS mRNA were expressed, and cold stimulation failed to increase iNOS mRNA levels in IBAT. Our previous results are in accordance with their findings, but we also observed that L-arginine treatment of cold-acclimated rats increases iNOS expression [21]. It is possible that L-arginine treatment synergistically with cold changes expression pattern of NOSs and, at least in some part may stimulate leptin expression observed in that experimental group.

In rats acclimated to cold (45 days), brown adipocytes show characteristic multilocularity and innervation are very dense. It is known that the SNS plays an essential role in the control of UCP1 gene expression [29]. Cold exposure activates thermogenesis by increasing noradrenaline release from sympathetic nerves, which induces UCP1 expression trough the β_3 adrenergic receptor-mediated and cAMP-dependent mechanism [18,21,30-33]. Simultaneously, also acting through the β_3-adrenergic receptor-mediated and cAMP-dependent mechanism, sympathetic stimulation inhibits leptin expression in brown and white adipose tissues [34].

However, leptin/UCP1 interrelationship is not so simple. Numerous studies indicate that leptin actually regulates UCP1 expression in IBAT [35-37] mainly through hypothalamic pathways [38]. In addition, leptin induction of UCP1 gene expression depends on sympathetic innervation [8,34,39]. Furthermore, Commins *et al.* [40] using β_3-receptor knockout mice, show that central leptin regulates UCP1 gene expression via different β-adrenoreceptor subtypes.

A question then arises about the necessity for suppressing leptin expression in a cold environment. Leptin is a hormone whose plasma levels are directly related to the amount of body fat [41,42]. Leptin, therefore, informs the central nervous system of the fat store size, which is in agreement with the adipostatic theory proposed by Kennedy [43]. To carry out adipostatic control, leptin affects both sides of the energy balance equation.

An adaptive increase in thermogenic activity may provide a mechanism for defending rats in a cold environment, but at the same time this hypermetabolic state lowers fat stores and animals respond with a compensatory hyperphagia. Thus, an increase in thermogenic activity may also provide a mechanism to preserve body weight by matching rates of energy utilization with energy intake [44].

The effects of leptin are quite different between animals acclimated to room temperature and to cold. In animals acclimated to room temperature, leptin administration increased their body temperature, basal metabolic rates, nonshivering thermogenesis and UCP1 expression in IBAT, indicating a potential involvement of leptin in thermogenesis [7,11]. In contrast to this, in cold acclimated rats, leptin administration reduced IBAT thermogenesis [45], and low serum leptin levels were accompanied by an increase in UCP1 gene expression [46].

Our results show that L-arginine treatment changes the leptin expression pattern observed in cold. Since we previously found that L-arginine additionally increases UCP1 expression in cold acclimated rats [21], it is possible that the leptin expression observed in brown adipocytes induced by L-arginine might be a stop signal for the overstimulated IBAT thermogenesis (energy expenditure higher than energy deposition). Locally, leptin may be stimulated by NO to oppose the effects of sympathetic activation and prevent overheating and/or underweight. Until now, there is no data for in vivo co-expression of leptin and UCP1 in thermogenically active brown adipocytes. We predict that UCP1 and leptin may be regulated in opposite directions by common factors involved in energy homeostasis, because of their inverse relationship with obesity and cold acclimation, and that NO overproduction may promote energy expenditure and weight loss in parallel.

Finally, we show that L-NAME treatment at room temperature leads to a diminishing leptin-immunopositivity of brown adipocyte cytoplasm. Moreover, we found that brown adipocyte nuclei, instead of cytoplasm, are strongly positive for leptin.

Nuclear positivity of brown adipocytes for some proteins has recently been revealed. Giordano *et al.* [16] showed existence of a noradrenalin-modulated functional NOS system in brown adipocytes nuclei. We also recently reported the nuclear positivity of brown adipocytes for iNOS [21].

Leptin has been shown to induce the release of NO [47-49] but, to our knowledge, no data regarding these leptin effects on NO in brown adipocytes have been reported so far.

It is of interest to note that L-NAME administration at 22 ± 1°C induces greater innervation than L-arginine treatment. It could be speculated that "unexpected" L-NAME effect on the innervation, partly the same as L-arginine effects, interfere with other metabolic effects of this amino acid analog. Using different

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model systems, Nagase *et al.* [50] and Moroz *et al.* [51] pointed to controversial L-NAME effects and surprising physiological response. The same authors emphasized that this L-NAME effect could not be connected to the inhibition of NO synthesis. Furthermore, they postulated that conflict observations in experiments with NOS inhibitors (L-NAME), can be the consequence of non-enzymatic production of NO-dependent species. This production was even connected to physiological, as well as numerous pathological effects when L-NAME was used. Some of these aspects could be responsible for the similarities of L-arginine and L-NAME effects, observed in the present study.

This finding also suggests that NO plays a key role in IBAT homeostasis at thermoneutrality, as well as in thermogenesis. It is possible that NOS inhibition at 22 ± 1°C induces sympathetic overactivity. This assumption is supported by the fact that Sherrer and Sartori [52] have documented that impaired NO synthesis is associated with sympathetic overactivity in insulin resistant states. It is possible that NOS inhibition at 22 ± 1°C causes imbalance between the central neural sympathoinhibitory and sympathoexcitatory actions of leptin. Leptin may be an important modulator of the sympathetic tone.

In conclusion, our results show that NO modulates leptin immunopositivity and innervation in IBAT. The effects observed depend on ambient temperature, e.g. tissue thermogenic function. Further studies using specific inhibitors of NOSs and measuring NO concentrations are needed for resolving the mechanism by which NO modulates leptin expression and innervation in IBAT.

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