

Thermoregulatory effect of alarin, a new member of the galanin peptide family

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Keywords: alarin, galanin peptide family, energy balance, body temperature, metabolic rate, heat loss

Abbreviations: Ta, ambient temperature; BW, body weight; Tc, core temperature; DMN, dorsomedial nucleus of the hypothalamus; FI, food intake; GALP, galanin-like peptide; HLI, heat loss index; ICV, intracerebroventricular; IP, intraperitoneal; MR, metabolic rate; NPY, neuropeptide Y; VO₂, oxygen consumption; PVN, paraventricular nucleus; PFS, pyrogen-free saline; Ts, tail skin temperature

In the background of obesity, among other factors, regulatory alterations in energy balance affecting peptide systems may also be assumed. Regulation of energy balance does not only involve maintenance of body weight but also that of metabolic rate and core temperature. The contribution of alarin, a new member of the potentially orexigenic galanin peptide family, to the regulation of energy metabolism has been recently suggested. Our aim was to analyze the thermoregulatory effects of alarin in rats.

Adult male Wistar rats received full-length alarin (alarin 1–25), its truncated form (alarin 6–25Cys) or scrambled alarin in various doses intracerebroventricularly at different ambient temperatures. Oxygen consumption, heat loss (assessed by tail skin temperature) and core temperature of rats were recorded in an indirect calorimeter system.

Upon alarin injection at 25 °C, an increase in oxygen consumption and continuous tail skin vasoconstriction induced a slow rise in core temperature that reached 0.5 °C by 120 and 1.0 °C by 180 min. At cooler or slightly warmer temperatures similar responses were seen. Neither the truncated nor the scrambled alarin elicited any significant thermoregulatory response, however, the truncated form antagonized the hyperthermic actions of the full-length peptide.

Alarin appears to elicit a slow hypermetabolic, hyperthermic response in rats. Such a thermoregulatory response would characterize a catabolic (anorexic and hypermetabolic) mediator. Further investigations are needed to clarify the complex role of alarin in energy homeostasis.

Introduction

Energy balance involves regulation of body temperature via heat production (metabolic rate, MR) and heat loss and that of body weight (BW) via food intake (FI) and MR. Anabolic substances lead to weight gain by increasing FI (orexigenic effect) and suppression of MR (hypometabolism, usually leading to hypothermia), while catabolic mediators act in an opposite way inducing anorexia, hypermetabolism and hyperthermia.¹ Energy balance appears to be positive (anabolic) until middle-age inducing weight gain, but with further aging, it turns negative (catabolic) resulting in loss of active tissues. Similar trends are seen in humans and other mammals, thus these age-related alterations of energy balance may be of regulatory origin also involving regulatory peptide systems.²

The regulatory role of some members of the galanin peptide family, such as that of galanin and galanin-like peptide (GALP) has been recently suggested in energy homeostasis.³ GALP

is a neuropeptide that has complex actions on energy balance, producing orexigenic effects⁴ in the short term in rats, but anorexigenic and febrile effects over the longer-term in rats and mice.^{5–7} Thus, food intake-related and thermoregulatory actions of GALP appear to be contradictory.⁸

The galanin peptide family has a new member (discovered in 2006) called alarin, named after the C-terminal residue alanine and its N-terminal residue serine. Alarin is a splice variant of the GALP gene excluding exon 3. It was first identified in gangliocytes of human neuroblastic tumors.⁹ Alarin has also been shown to be localized around blood vessels in the skin and it plays a role in the inhibition of neurogenic inflammation caused by substance P.¹⁰ In addition, it increases the secretion of luteinizing hormone in male mice.¹¹

Some data suggest that alarin is an orexigenic peptide. It was shown that an acute intracerebroventricular (ICV) alarin injection increased the FI and BW after 24 h in male mice.¹¹

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Submitted: 06/02/2014; Revised: 06/30/2014; Accepted: 06/30/2014; Published Online: 07/02/2014
<http://dx.doi.org/10.4161/temp.29790>

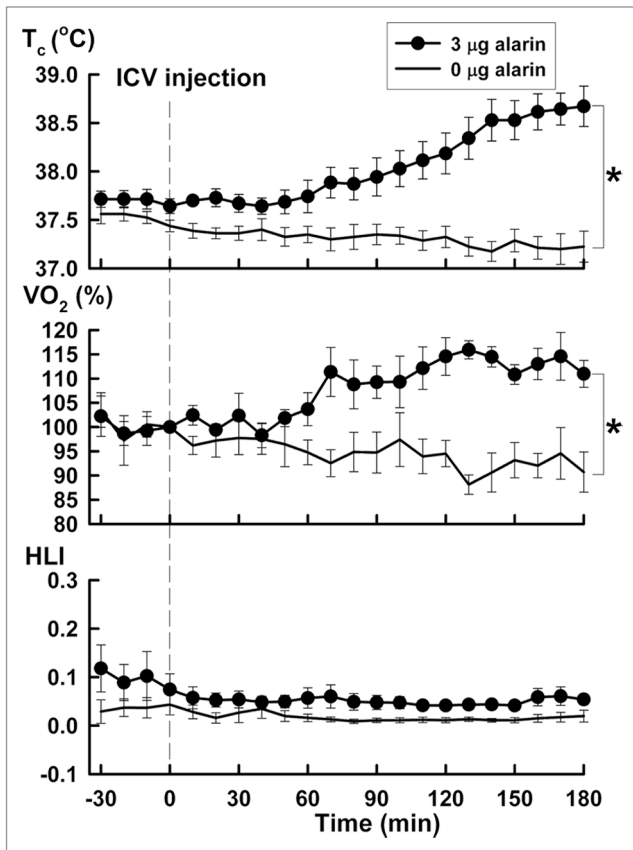


Figure 1. Thermoregulatory effects of an ICV injection of 3 µg alarin in young adult male Wistar rats at 25 °C ambient temperature. Upper panel shows changes in core temperature (T_c) ($F_{(1,13)} = 28.005, P < 0.001$); middle panel shows changes in oxygen consumption (VO_2) in percentage of the initial value (21.42 ± 1.84 vs. 22.01 ± 1.35 ml/kg/min for 3 vs. 0 µg alarin) ($F_{(1,13)} = 21.585, P < 0.001$); lower panel shows the heat loss index (HLI). Asterisks indicate significant differences.

Elimination of the first five amino acids of alarin abolished this effect, moreover the fragment also showed specific antagonistic activity.¹² A significant FI stimulating effect of full-length alarin was also described in male rats.^{13,14} Because of its orexigenic actions an anabolic character, hypothermic thermoregulatory actions of this peptide may be assumed.

Although its acute orexigenic action has been described in rats, its complex contribution to the regulation of energy homeostasis also involving thermoregulation is still unknown. Our present study focused on the analysis of the thermoregulatory role of alarin.

Results

An acute ICV injection of 3 µg full-length alarin elicited a delayed significant increase in oxygen consumption (VO_2) at 25 °C (Fig. 1). This hypermetabolism and concurrent continuous tail skin vasoconstriction, as shown by the low value of the heat loss index (HLI, see “Methods”), induced a slow but significant rise in core temperature (T_c) that reached 0.5 °C by 120, and 1.0 °C by 180 min after the injection. HLI, calculated

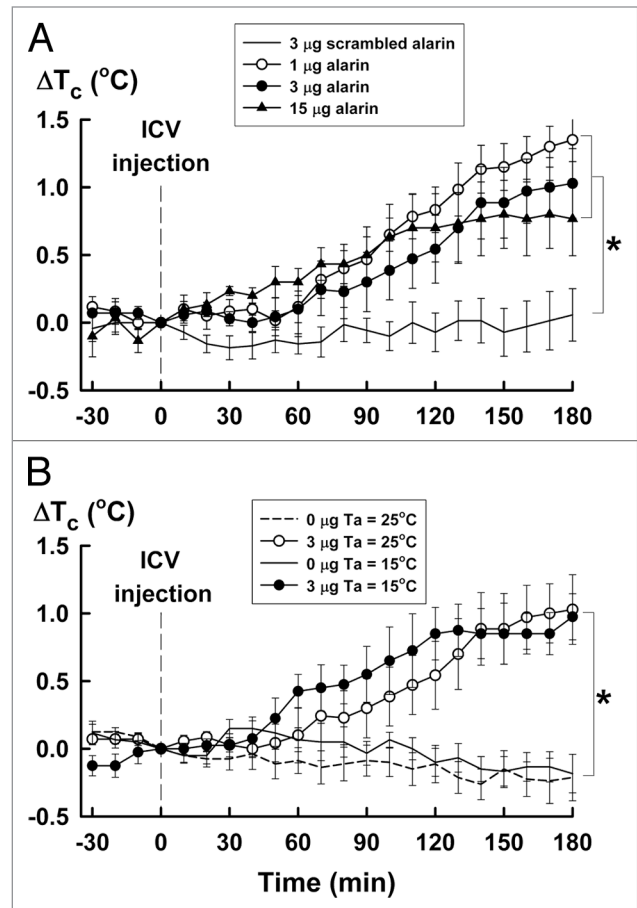


Figure 2. (A) Changes in core temperature (ΔT_c) upon ICV administration of different doses of alarin in young adult male Wistar rats at 25 °C ambient temperature as compared with the respective initial values ranging from 37.38 to 37.64 °C (the mean initial T_c values were similar in all groups). The hyperthermic response was significant for each dose as compared with the effects of scrambled alarin. (1 µg: $F_{(1,11)} = 20.483, P = 0.001$; 3 µg: $F_{(1,12)} = 6.428, P = 0.026$; 15 µg: $F_{(1,8)} = 9.567, P = 0.015$). Asterisks indicate significant differences. (B) Changes in core temperature (ΔT_c) upon ICV administration of 3 or 0 µg alarin at 15 or 25 °C ambient temperatures. Initial core temperatures range from 37.42 to 37.70 °C (the mean initial T_c values were similar in all groups). For 25 °C 0 vs. 3 µg: $F_{(1,12)} = 27.454, P < 0.001$; for 15 °C $F_{(1,8)} = 13.731, P = 0.006$. Asterisks indicate significant differences.

according to the literature, ranges between 0 and 1. As the value approaches 0—vasoconstriction, when the value approaches 1—vasodilation is indicated.

Different doses of alarin (1, 3, or 15 µg) induced significant hyperthermias at 25 °C (Fig. 2A), i.e., no dose-dependence was observed. Scrambled alarin failed to induce any response (Fig. 2A). At cooler ambient temperatures (15 °C [Fig. 2B] or 20 °C [not shown]) the administration of 3 µg alarin resulted in similar T_c rises as those seen at 25 °C.

Above the vasodilation threshold (28 °C) a pronounced alarin-induced vasoconstriction was accompanied by a significant hyperthermic effect as shown in the individual recording of Figure 3. Upon peripheral injections, full-length alarin failed to induce hyperthermia or vasoconstriction (not shown).

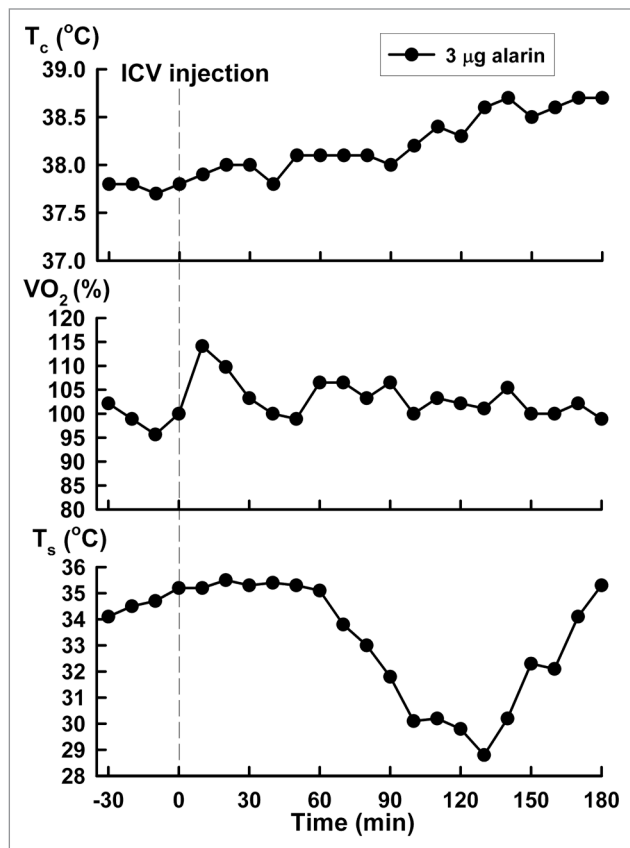


Figure 3. Individual recording of the rise in core temperature (T_c) (upper panel), in oxygen consumption (VO_2) in percent of the initial value (middle panel, initial value 20.75 ml/kg/min) and the fall in tail skin temperature (T_s) indicating vasoconstriction (lower panel) upon ICV injection of 3 μ g alarin at 28 °C ambient temperature.

According to earlier data,¹¹ elimination of the first 5 amino acids of alarin (alarin 6–25Cys, 2.5 μ g) abolished the orexigenic action, i.e., resulted in an antagonistic effect. In our experiments, upon central administration truncated alarin did not elicit any significant thermoregulatory response.

Truncated alarin, when administered together with ICV full-length alarin (3 μ g), abolished the hyperthermic action of full-length alarin (Fig. 4). Thus an antagonistic effect of alarin 6–25Cys was demonstrated.

Discussion

The present study investigated the thermoregulatory effects of alarin, a 25 amino-acid peptide, the newest member of the orexigenic galanin peptide family that shows structural and functional similarities to 60-amino-acid GALP.^{11,12,15,16} Alarin immunoreactivity shows a broader expression pattern in the murine brain than that of GALP, including different nuclei of the hypothalamus (dorsomedial nucleus of the hypothalamus [DMN], lateral hypothalamus, paraventricular nucleus [PVN]).¹⁶ Kofler and coworkers recently reported that an ICV injection of alarin significantly increased the expression of the immediate early gene *c-fos*, a marker for neuronal activation in different

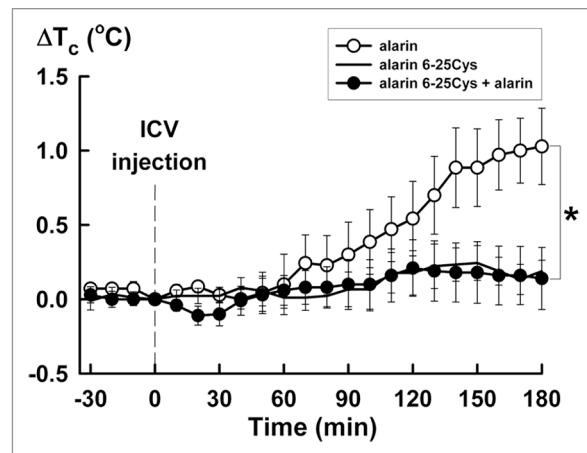


Figure 4. Changes in core temperature (ΔT_c) upon ICV administrations of alarin, truncated alarin (Ala6–25Cys) or an alarin + Ala6–25Cys combination in young adult male Wistar rats at 25 °C ambient temperature. The hyperthermic effect of full-length alarin significantly differed from that of the truncated form ($F_{(1,13)} = 14.550$, $P = 0.002$) or from that of the combined administration ($F_{(1,14)} = 7.624$, $P = 0.015$). Initial core temperatures ranged from 37.42 to 37.65 °C and were similar in all groups. Asterisk indicates significant differences.

brain regions including the PVN, DMN, and the arcuate nucleus of male rats.¹⁴ Alarin also induces neuropeptide Y (NPY) release from hypothalamic explants similar to GALP, suggesting that alarin may mediate its effect on feeding via similar pathways.¹³ However, no conclusive evidence (such as successful application of NPY antagonists to inhibit alarin effects) have been proposed for NPY mediation of alarin actions.

As alarin (unlike GALP) has no detectable affinity toward any of the known three galanin receptor subtypes,^{10,13} its actions are assumed to be mediated by a separate or an as yet unidentified receptor.

Although a previous report failed to reveal any change in body temperature upon an ICV alarin injection in freely moving mice using biotelemetry,¹¹ and no change in oxygen consumption has been detected in adult male freely moving Long-Evans rats upon a similar alarin administration either (in freely moving rats locomotion itself may raise metabolic rate¹⁴), as yet no conclusive thermoregulatory tests involving alarin have been conducted in rats.

The present data show that centrally administered alarin appears to elicit a slow but significant hypermetabolic, hyperthermic thermoregulatory response, further enhanced by a suppression of heat loss in rats. The rate of this thermoregulatory response is similar at different doses and at a wide range of ambient temperatures (from 15 to 28 °C). Similarly to food intake-related observations,¹² the thermoregulatory actions of alarin were also lost when the first 5 amino acids were removed, and the truncated peptide also acted as an antagonist in our thermoregulatory tests.

Based on the long latency of the central hyperthermic response and on previous reports about cutaneous vasoconstriction induced by peripheral alarin injections,¹⁰ the question arises whether the observed delayed thermoregulatory effects of

full-length alarin are partly due to its peripheral actions following passage of the peptide from the lateral ventricle to the peripheral circulation. However, our data show that direct peripheral IP administration of the peptide at doses used also ICV failed to show any thermoregulatory response.

With regard to the potential NPY mediation of alarin effects, the thermoregulatory effects of alarin appear to differ from those of NPY in a cool environment, where acute ICV NPY administration elicits acute hypometabolism and hypothermia before leading to some delayed rise in body temperature. Moreover, NPY does not induce vasoconstriction within a similar range of ambient temperatures.¹⁷

The thermoregulatory effects of alarin described in our study appear to be somewhat similar to those of GALP. Upon acute ICV GALP injection body temperature rises promptly lasting for 6–8 h in rats.⁸ So far, GALP seems to elicit a fever-like hyperthermia,¹⁸ because it appears to be mediated by interleukin-1 and prostaglandins^{8,19} and hypermetabolism is also associated with a reduced heat loss.²⁰ Similarities between the thermoregulatory effects of alarin and GALP and also the delay in the onset of alarin-hyperthermia raise the potential involvement of prostaglandins as secondary mediators in the responses elicited by alarin.

Although alarin is a member of the orexigenic and presumably anabolic galanin peptide family, the thermoregulatory responses to alarin described in our study (and those to GALP) could rather characterize catabolic peptide mediators. These data suggest therefore that the complex role of alarin in the regulation of energy homeostasis may have to be carefully re-evaluated including further investigations focusing on its acute and long-term effects on food intake, metabolic rate and body weight. Further studies are required to identify the different factors that regulate alarin release and those mediating its effects.

Materials and Methods

Animals

For the experiments 3 mo old male Wistar rats of 340–360 g initial BW were used from the animal colony of the Department of Pathophysiology and Gerontology, University of Pécs. They were maintained at an ambient temperature of 23–26 °C. The lights were on between 6 and 18 h. The rats were kept individually in plastic cages with wood-chip bedding and they had standard laboratory rat chow (CRLT/N rodent chow, Szindbád Kft., 11 kJ/g) and water ad libitum. All animals were habituated to regular handling and were familiarized for at least a week prior to experiments to semi-restraining cylindrical cages that were used later on during the analysis of MR and body temperature (T_c).

All experimental interventions and procedures were performed by strictly observing the general rules and following the special permission of the University of Pécs Ethical Committee for the Protection of Animals in Research (BA 02/2000–11/2011, valid for 5 y). In general, the rules of this Committee are in accord with the main directives of the European Committees Council (86/609/EEC, Directive 2010/63/EU of the European Parliament and of the Council).

Surgeries

After about 1 wk adaptation period in the laboratory, the right lateral cerebral ventricle was stereotaxically cannulated (parameters: 1 mm posterior and 1.5 mm right lateral to bregma, 3.5 mm ventral to dura) for the purpose of ICV injections. Surgical interventions were performed under intraperitoneal (IP) ketamin + xylazine (78 mg/kg [Calypsol, Richter] + 13 mg/kg [Sedaxylan, Eurovet]) anesthesia. Animals also received 2 mg IP gentamycin. The 22-gauge metal leading cannula was fixed with the help of two miniature screws and dental cement. This guide cannula was usually closed by a stylet, except during tests. A few days after the implantation of this cannula for the testing of its location the stylet was removed and replaced by a fitting 29-gauge injection cannula outreaching the guide cannula by about 0.5 mm. Through a pp10 polyethylene tube attachment angiotensin II (Sigma, A9525, 20 ng/5 μ l) was injected ICV and the subsequent water consumption was measured. The location of the cannula was assumed to be appropriate and the test was positive if at least 5 ml water was consumed within 30 min. For IP injections, before the tests, a polythene tube was acutely inserted into the abdominal cavity through a needle under ether anesthesia; after withdrawal of the needle, the tube tunneled under the skin was exteriorized at the nape. Following experiments the animals were euthanized by IP urethane (3–5g/kg, Reanal). Brains were removed and fixed, the injection sites were checked macroscopically by coronal sections. Rats with inappropriately located cannula were excluded from the analysis.

Substances applied

Synthetic full-length alarin (alarin 1–25, MW: 2820.19), its truncated form (alarin 6–25Cys, MW: 2389.74) and a scrambled variant were custom synthesized by GL Biochem. All peptides were dissolved in pyrogen-free saline (PFS) and given in 5 μ l volume ICV. To test dose dependence, full-length alarin was administered at doses of 1, 3, or 15 μ g at 9 h (i.e. early in the inactive phase of the circadian activity) at various environmental temperatures. In these experiments all doses were given to the same group of animals, but the order of doses and that of ambient temperatures were randomized. At least five days elapsed between such tests. In other tests alarin 6–25Cys (2.5 μ g) was administered with or without full-length alarin (3 μ g) to investigate its potential antagonistic thermoregulatory effects. We also tested the effects of a scrambled alarin (3 μ g) containing the same amino acids as alarin, in random order. In the experiments different doses of the peptides filled a 5 μ l volume of the proximal end of the 20–25 cm-long pp10 polyethylene tube that was attached to the injection cannula, while the rest of the tube was filled with PFS. A small bubble separated the substance from the PFS in the distal part of the tube. Injecting 5 μ l saline at the distal end of the tube resulted in remote ICV delivery of the substance. To test the potential peripheral actions of the applied doses of full-length alarin, they were also administered IP.

Measuring thermoregulatory functions

In metabolic chambers of an Oxymax indirect calorimeter oxygen consumption (VO_2) and carbon-dioxide production (to determine MR), as well as an indicator of heat loss [tail skin temperature (T_s), representing tendencies of changes in heat

loss function], and the resulting core body temperature (T_c) measured in the colon were assessed.

Our indirect calorimeter measures gas concentrations and flow. Flow is determined by a mass thermal transfer technique that yields data formatted in terms normalized to scientific STPD (i.e. standard temperature and pressure, dry: 0 °C and 760 mmHg, removal of water vapor by materials with hygroscopic properties that isolate the sample gas from the drying media).

Measurements took place between 9 h and 15 h, and during this period the animals could not eat or drink. The rats were semi-restrained in a cylindrical wire-mesh confiner in which they could not turn around but their other movements were not prevented. Having been accustomed to the confiner, semi-restriction did not cause particular stress to the animals. Together with this cylinder, they were placed into an open-circuit metabolic chamber, which in turn, was immersed into a thermostatically controlled water-bath to secure a standard (from thermoneutral to cold) ambient temperature (T_a) in the chamber. At a T_a of 28 °C regularly vasodilation is observed, at 25 °C there is vasoconstriction and no fluctuations in tail skin temperature (concept of thermoneutrality by Romanovsky et al. 2002²¹), but—in contrast to the cold 20 or 15 °C—they allow to evoke either skin vasodilation or heat loss. On the other hand, at the colder T_a values the cold-induced hypermetabolism allows the observation of a possible hypothermic effect. Four metabolic chambers were used simultaneously. Copper-constantan thermocouples were attached to the animals for measuring temperatures: one was introduced into the colon (at least 10 cm from the anus, fixed by tape to the tail) for measuring T_c and another one was placed and fixed onto the dorsal skin of the distal part of the tail for measuring T_s . T_a was measured simultaneously. All temperature data were collected by a Digi-Sense Benchtop scanning thermometer (Cole

Parmer) for electronic evaluation. Heat loss state (“heat loss index,” HLI, as used in earlier studies²²) was assessed from the relationship of the three monitored temperatures ($HLI = [T_s - T_a] / [T_c - T_a]$): T_s values approaching T_a (HLI near 0) suggested vasoconstriction as a sign of heat conservation state, while those values nearer to T_c (HLI near 1) suggested vasodilation as an early manifestation of general enhancement of heat loss activity. The thermocouples and the extension of the ICV or IP cannula were pulled through a tightly sealed opening of the chamber, enabling us to inject the animals without causing them any acute disturbance or discomfort.

Total heat production was judged from the metabolic rate measured by indirect calorimetry, on basis of gas analysis of the air samples leaving the ventilated chamber and expressed as VO_2 in ml O_2 /kg/min.

Statistical analysis

Test groups contained at least 6–8 rats. Repeated-measures ANOVA using SPSS for Windows 11.0 software was applied for the statistical analysis of the data. Differences were accepted as statistically significant at the level of $P < 0.05$. Mean \pm SEM are indicated in all figures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Financial support: FFG, 822782/THERAPEP, Hungarian Scientific Research Fund: OTKA PD84241, Grants of the University of Pécs: 34039/KA-OTKA/13–02, 13–25.

The excellent, expert technical help at different parts of this study provided by Ms. A. Jech-Mihálffy, Ms. M. Gáspár-Koncsecskó, and Ms. A. Bóka-Kiss is gratefully acknowledged.

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