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Naturally occurring hypothermia promotes survival in severe anaphylaxis

Ricardo W. Alberca, Eliane Gomes, Eduardo H. Moretti, Momtchilo Russo & Alexandre A. Steiner*

Departamento de Imunologia, Instituto de Ciencias Biomedicas, Universidade de Sao Paulo, Sao Paulo, SP 05508, Brazil

*Correspondence: asteiner@usp.br

Highlights

- Adjustments in body core temperature may be lifesaving in the face of adverse environmental or physiological conditions.
- Here we show that naturally occurring (spontaneous) hypothermia promotes survival in severe cases of ovalbumin-induced anaphylaxis in mice.
- Development of hypothermia improved oxygenation of the brain and kidneys in this model, without affecting mast cell degranulation.
- The perception that hypothermia reflects dysregulation in anaphylaxis may need to be revisited.

Abstract

Although hypothermia has received substantial attention as an indicator of severity in anaphylaxis, it has been neglected from the perspective of whether it could act as a disease-modifying factor in this condition. Here, the impact of naturally

occurring (spontaneous) hypothermia on anaphylaxis was evaluated in a murine model of ovalbumin (OVA)-induced allergy. Nonextreme changes in the ambient temperature (T_a) were used to modulate the magnitude of spontaneous hypothermia. At a T_a of 24°C, challenge with OVA intraperitoneally or intravenously resulted in a rapid, transient fall in body core temperature, which reached its nadir 4-6°C below baseline in 30 min. This hypothermic response was largely attenuated when the mice were kept at a T_a of 34°C. The T_a -dependent attenuation of hypothermia resulted in a survival rate of only 30%, as opposed to survival of 100% in the condition that favored the development of hypothermia. The protective effect of hypothermia did not involve changes in the rate of mast cell degranulation, as assessed by the concentration of mast cell protease-1 in bodily fluids. On the other hand, hypothermia improved oxygenation of the brain and kidneys, as indicated by higher $NAD^+/NADH$ ratios. Therefore, it is plausible to propose that naturally occurring hypothermia makes organs more resistant to the anaphylactic insult.

Keywords

anaphylactic shock; allergy; body temperature; thermoregulation; oxygenation; hypoxia.

1. Introduction

A decrease in body core temperature (T_c), hypothermia, is a hallmark of anaphylaxis in experimental rodents [1-3], and has been reported in children suffering from severe food allergy [4]. Within minutes of an allergen challenge in mice, bioactive substances released by mast cells, including histamine, mediate the fall in T_c [5-8]. Such hypothermia is generally viewed as a failure to maintain body temperature, mainly on the basis of its association with the severity of circulatory shock [1-3].

However, association does not imply in causality, and there are at least two reasons to suggest that hypothermia may be more than a shock-induced system failure in anaphylaxis. First, whereas intravenous albumin prevents hypovolemia and shock, it exerts only a minor effect on the development of hypothermia in a murine model of IL-4/PAF-dependent anaphylaxis [9]. Second, the effects of histamine on T_c are much more complex than what would be expected in a situation of thermoregulatory failure, with brain-mediated autonomic and behavioral effects having been reported [10, 11]. This thermoeffector profile resembles that of regulated thermoregulatory responses with potential adaptive value [12], such as those that accompany life-threatening cases of hypoxic hypoxia [13] and sepsis [14-16].

In this setting, the present study was designed to test the hypothesis that naturally occurring (spontaneous) hypothermia may be of value in a murine model of OVA-induced severe allergy. Nonextreme changes in the ambient temperature (T_a) were employed to modulate the magnitude of the T_c change. Survival rate, mast cell degranulation and tissue oxygenation were evaluated.

2. Materials and Methods

2.1. Animals

The study was conducted in 6-8 week-old female C57BL/6J mice obtained from the pathogen-free facility of the Institute of Biomedical Sciences (University of Sao Paulo, São Paulo, Brazil). C57BL/6J mice have been shown to be as susceptible as BALB/c mice in severe anaphylactic reactions that involve not only IgE but also IgG1-

specific antibodies [17-19]. What is more, in a model of peanut extract-induced anaphylaxis, C57BL/6J mice have been shown to mount more pronounced hypothermic responses than BALB/c mice [20].

The animal room was under a 12:12 h light-dark cycle (lights on at 7:00 AM), with room temperature maintained between 24°C and 27°C. Filtered water and standard chow were available *ad libitum*. All protocols were approved by the Animal Care and Use Committee at the Institute of Biomedical Sciences of the University of São Paulo (Sao Paulo, Brazil).

2.2. Allergic sensitization protocol

Mice were sensitized with two subcutaneous (s.c.) doses of ovalbumin (OVA, 4 µg) in alum gel (1.6 mg), on *days 0* and *7*, respectively. Boosts with intra-nasal OVA (10 µg) were made on *days 14* and *21*. The experiments were conducted on *day 28*, when the mice were challenged with 100 µg of OVA intranasally, subcutaneously, intraperitoneally, or intravenously (orbital vein). This protocol has been validated and described in detail elsewhere [21].

OVA was obtained from Sigma-Aldrich and cleared of any lipopolysaccharide-related impurities by four washes with Triton X-114. The content of lipopolysaccharide in the purified OVA preparation (5 mg/ml) was less than 0.02 units/mg. Alum, a gel of Al(OH)₃, was prepared by the precipitation of AlH₄(SO₄)₂·12 H₂O with an excess of NaOH. The product was washed with ultrapure water five times before use.

2.3. Experimental set-up

On the day of the experiment, the mice were kept individually caged in an environmental chamber (NQ1 model; Environmental Growth Chambers), which

provided precise control of the ambient temperature (T_a). After two hours of habituation to the experimental environment, the mice were challenged with OVA and maintained at a T_a of 24°C or 34°C. This range of T_a is associated with neither heat nor cold stress in mice, but is typically sufficient to allow or prevent spontaneous forms of hypothermia from developing [22].

2.4. *Thermometry*

Colonic temperature was used as an index of body core temperature (T_c). The measurement was made using a petroleum jelly-coated thermocouple, inserted 2 cm from the anal sphincter. To minimize the stress associated with the procedure, each mouse had been thoroughly habituated to this procedure during the week that preceded an experiment.

2.5. *Sample harvesting*

At the time point of interest, mice were euthanized by cervical dislocation immediately prior to sample harvesting. Blood was collected by cardiac puncture; the blood was allowed to clot for 20 min at room temperature, after which serum was separated by centrifugation ($350 \times g$, 5 min). Peritoneal lavage was performed with 3 ml of ice-cold PBS. Bronchoalveolar lavage fluid was collected by flushing the lungs (via the trachea) in and out with 1 ml of ice-cold PBS. Brain, kidney and liver were dissected out in their entirety, and immediately snap frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

2.6. *ELISA*

OVA-specific immunoglobulin isotypes (IgE, IgG1 and IgG2c) and mast cell protease-1 (MCPT-1, a marker of mast cell degranulation [23]) were assayed by sandwich ELISA using reagents and protocols from Chondrex and Invitrogen, respectively.

2.7. NAD^+/NADH determination

The contents of NAD^+ and NADH in tissue samples were determined by a cycling enzyme method from BioVision, as described elsewhere [15].

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism V8. Survival analysis was performed using the log-rank (Mantel-Cox) test. T_c was evaluated across groups and time points by repeated-measures ANOVA. One-way ANOVA was used when MCPT-1 concentrations were compared across more than two independent groups. Student's *t*-test was employed for pairwise comparisons of immunoglobulin titers, MCPT-1 concentrations and NAD^+/NADH ratios. The level of significance was set at $p < 0.05$.

3. Results and Discussion

The murine model of OVA-induced allergy employed herein is known to involve not only IgE, but also IgG1-specific anaphylactic antibodies [17-19]. Accordingly, the sensitized mice in the present study had high serum titers of OVA-specific IgE and IgG1 (Fig 1A-B), but undetectable titers of OVA-specific IgG2c (Fig. 1C).

Mice were challenged with OVA via distinct routes of administration at a T_a of 24°C; control mice received phosphate-buffered saline (PBS). Hypothermia did not develop when the mice were challenged with OVA via the intranasal or subcutaneous route (Fig. 1D-E). However, hypothermia developed when OVA was delivered via a quick absorption route (intraperitoneal, Fig. 1F) or via the intravenous route (Fig. 1G). This finding may be interpreted as indication that hypothermia develops when the allergic reaction reaches a systemic level. Accordingly, the mast cell degranulation marker, MCPT-1 [23], significantly rose in the serum of the mice challenged intraperitoneally or intravenously, but not in the serum of the mice challenged intranasally or subcutaneously (Fig. 1H). Assessment of MCPT-1 in the bronchoalveolar lavage fluid further indicated that a local reaction was induced by the intranasal challenge (Fig. 1I), which was not sufficient to cause hypothermia (Fig. 1D). We did not have samples to assess whether a local reaction took place with the subcutaneous challenge, but it seems unlikely that it would not have taken place. Indeed, MCPT-1 also rose locally in the peritoneum of the mice challenged with OVA intraperitoneally (Fig. 1J).

Because the intraperitoneal challenge resulted in the most pronounced response, it was employed in the subsequent experiments. Anaphylaxis-induced hypothermia was largely attenuated by exposure to a T_a of 34°C (Fig. 2A). Being typically near the upper limit of the thermoneutral zone for mice [22, 24], a T_a of 34°C is not extreme for this species. T_a s of 39-43°C are usually necessary to cause heat stress in mice [25, 26]. The T_a dependency of anaphylaxis-induced hypothermia over a nonextreme T_a range appears to be a common characteristic of spontaneous forms of hypothermia in rats and mice, having been reported with respect to the hypothermic responses that accompany hypoxia [27] or endotoxic shock [14, 15, 28]. The reason underlying this characteristic

is thought to be that hypothermia in these cases relies more on a regulated suppression of thermogenesis (operant a lower T_a s) than on resetting of heat-loss effectors (operant a higher T_a s) [28]. At least in the case of endotoxic shock, increasing T_a does not attenuate the production of the inflammatory mediators known to cause hypothermia [14, 29].

In the present study, we took advantage of this T_a dependency to assess whether and how development of hypothermia impacts the outcome of i.p. OVA-induced anaphylaxis. Mice not allowed to develop hypothermia at the T_a of 34°C had a survival rate of only 30%, compared to a survival rate of 100% in the mice that were allowed to develop hypothermia at the T_a of 24°C (Fig. 2B). Therefore, like in severe cases of hypoxia and sepsis [14, 16, 27], natural development of hypothermia promotes protection in severe anaphylaxis.

It is important to note that allowing or preventing naturally occurring forms of hypothermia using nonextreme changes in T_a is quite distinct from using forced hypothermia, which consists of applying severe cooling to lower T_c to a target level that is typically much lower than that attained during natural, spontaneous forms of hypothermia [30]. It is also distinct from the approaches employed to evaluate how long-term changes in housing temperature, usually for weeks or months, may influence physiology in health and disease without affecting T_c itself [31, 32].

Regarding the mechanisms involved in the thermal modulation of anaphylaxis-associated mortality, we observed that the T_a -dependent prevention of spontaneous hypothermia did not impact the rate of mast cell degranulation, as assessed by MCPT-1 secretion in serum and peritoneal lavage (Figs. 2C-D). The implication of this observation is twofold. First, it concurs with the notion that the T_a -dependent suppression of hypothermia does not involve attenuated secretion of its mediators, which in the case of anaphylaxis include histamine and possibly other substances

released by mast cells [5-8]. Hence, like in the case of endotoxic shock-associated hypothermia [14, 29], T_a is more likely to allow or prevent development hypothermia by influencing which set of thermoeffectors is operant, as discussed above. The second implication is that changes in the rate of mast cell degranulation are unrelated to the protection provided by hypothermia.

We then assessed whether the protective mechanism of hypothermia in anaphylaxis could involve mitigation of tissue hypoxia, a downstream event in the pathology of anaphylactic shock [3]. Using the $NAD^+/NADH$ ratio as an indicator of oxygen availability for mitochondrial respiration [33], we were able to identify the brain (Fig. 2E) and the kidneys (Fig. 2F) as organs that were better oxygenated when hypothermia was allowed to develop. This potential benefit was organ-specific, since hypothermia did not affect $NAD^+/NADH$ ratios in the liver (Fig. 2G). The mechanisms by which hypothermia can improve tissue oxygenation has been studied in endotoxic shock, and the available evidence indicates that a regulated decrease in T_c lowers oxygen demand more than oxygen delivery [15].

4. Conclusion and Perspectives

The present study provides evidence that development of hypothermia has a biological value in life-threatening cases of anaphylaxis, at least in a murine model of OVA-induced allergy. The data also indicate that alleviation of tissue hypoxia poses as a plausible mechanism for the protection provided by hypothermia, but future studies are certainly necessary to assess the mechanisms of protection in more depth.

Because a biological value is unlikely to be coupled with thermoregulatory failure, the perception that hypothermia reflects thermoregulatory failure in anaphylaxis ought to be revisited. Future studies are necessary to directly evaluate whether

anaphylaxis-induced hypothermia is in fact a regulated thermoregulatory response. Such studies must consider that our understanding of how the thermoregulatory system works has changed substantially over the past two decades. More specifically, the concept that all thermoeffectors are coordinated by a single controller has given way to the concept that each thermoeffector is driven by a parallel, independent neural pathway, each operating at a different temperature threshold and response gain [34, 35]. The main implication of this conceptual transformation is that it explains how regulated thermoregulatory responses can be achieved by many combinations of thermoeffector responses. For example, the regulated hypothermia induced by bacterial lipopolysaccharide has been shown to be brought about suppression of nonshivering thermogenesis in combination with cold-seeking behavior, without resetting of heat defense effectors [28].

The present study also paves the road for clinical studies on the thermal management of anaphylaxis. Hypothermia has been well documented in children suffering from food protein-induced enterocolitis syndrome, a non-IgE form of food allergy [4]. Perhaps allowing this spontaneous form of hypothermia to run its natural course could result in a better outcome. It will also be important to systematically evaluate whether hypothermia occurs in adults suffering from severe allergic episodes. If, as in mice, anaphylaxis-associated hypothermia happens to be short-lived and self-limiting in humans, its occurrence might have been overlooked or underestimated in clinical practice.

Conflict of interest

The authors declare that they do not have any conflict of interest, financial or otherwise.

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Figure 1. Immune profile and thermal responses in the murine model of OVA-induced allergy. (A-C) OVA-specific immunoglobulin (Ig) isotypes in the serum of sensitized vs. naïve mice. (D-G) Change (Δ) in the T_c of sensitized mice challenged

with OVA via different routes of administration: intranasal (i.n., D); subcutaneous (s.c., E); intraperitoneal (i.p., F); or intravenous (i.v., G). Time zero in the graphs corresponds to the time of the challenge, or to the control administration of PBS in sensitized mice. Basal T_c averaged $36.7 \pm 0.1^\circ\text{C}$, and did not differ between groups. **(H-J)** MCPT-1 concentrations in serum **(H)**, bronchoalveolar lavage fluid **(I)** or peritoneal lavage fluid **(J)** of sensitized mice challenged or not with OVA. The routes of OVA administration are indicated. In all panels, data are shown as means $\pm$ SEM. The number of animals per group was 4-5. * $p < 0.05$ and ** $p < 0.01$ compared to the corresponding control group; t-test was used for pairwise comparisons in A, B, C, I and J; repeated-measures ANOVA was used for statistical comparisons in D-G; one-way ANOVA was employed in H.

Figure 2. Preventing development of hypothermia by exposure to a warm environment decreases survival rate and worsens tissue oxygenation in OVA-induced anaphylaxis. (A-B) Impacts of nonextreme ambient temperatures (T_a s) on the hypothermic responses (A) and survival rates (B) of sensitized mice to an i.p. challenge with OVA. Basal T_c averaged $36.4 \pm 0.2^\circ\text{C}$, and did not differ between groups. **(C-D)** Concentration of MCPT-1 in peritoneal lavage (C) or serum (D) harvested at 20 min post-OVA from mice that were allowed (T_a of 24°C) or not allowed (T_a of 34°C) to develop hypothermia. This time point precedes any mortality in the group not allowed to develop hypothermia. **(E-G)** NAD^+/NADH ratios (an indicator of tissue oxygenation) in samples of brain (E), kidneys (F) and liver (G) harvested at 20 min post-OVA from mice that were allowed (T_a of 24°C) or not allowed (T_a of 34°C) to develop hypothermia. Data are shown as means $\pm$ SEM, with the exception of survival rate. The number of animals per group was 10 in A-B, 8-9 in C-D, and 3 in E-G. * $p < 0.05$ and ** $p < 0.01$ compared to the corresponding control group; repeated-measured ANOVA

was used for statistical comparisons in A; the Log-rank (Mantel-Cox) test was used to compare survival ratios in B; t-test was used for pairwise comparisons in C-G.



