Reduction of body temperature governs neutrophil retention in hibernating and nonhibernating animals by margination

Hjalmar R. Bouma,,1 *George J. Dugbartey,* *Ate S. Boerema,†* *Fatemeh Talaei,**
Annika Herwig,‡ Maaike Goris, *Azuwerus van Buiten,* *Arjen M. Strijkstra,†*
Hannah V. Carey,§ Robert H. Henning, *and Frans G. M. Kroese‖*

Departments of *Clinical Pharmacology and ‖Rheumatology and Clinical Immunology, University Medical Center Groningen, and †Centre for Behavior and Neuroscience, Departments of Chronobiology and Molecular Neurobiology, University of Groningen, Groningen, The Netherlands; ‡Zoological Institute, University of Hamburg, Hamburg, Germany; and §Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin, USA

ABSTRACT

Hibernation consists of periods of low metabolism, called torpor, interspersed by euthermic arousal periods. During deep and daily (shallow) torpor, the number of circulating leukocytes decreases, although circulating cells, is restored to normal numbers upon arousal. Here, we show that neutropenia, during torpor, is solely a result of lowering of body temperature, as a reduction of circulating also occurred following forced hypothermia in summer euthermic hamsters and rats that do not hibernate. Splenectomy had no effect on reduction in circulating neutrophils during torpor. Margination of neutrophils to vessel walls appears to be the mechanism responsible for reduced numbers of neutrophils in hypothermic animals, as the effect is inhibited by pretreatment with dexamethasone. In conclusion, low body temperature in species that naturally use torpor or in nonhibernating species under forced hypothermia leads to a decrease of circulating neutrophils as a result of margination. These findings may be of clinical relevance, as they could explain, at in least part, the benefits and drawbacks of therapeutic hypothermia as used in trauma patients and during major surgery. *J. Leukoc. Biol.* 94: 431–437; 2013.

Introduction

Hibernation is an energy-conserving mechanism that promotes survival of endothermic animals during periods of scarce food supply. The hibernation season is characterized by periods of reduced metabolism and body temperature known as torpor bouts, which are interspersed by short (<24 h) arousal periods to normal (euthermic) body temperatures. During bouts of deep torpor, which last for several days to a month, body temperature can fall as lows as low as ~3°C, depending on species and ambient temperature [1–5]. A second form of metabolic depression is "daily torpor", in which minimum body temperatures typically reach only 18–30°C, and torpor bout duration does not exceed 24 h [6]. In addition to a suite of physiological changes that occur during torpor (e.g., depressed heart and ventilation rates [7]), immune function is reduced significantly [8]. The innate and the adaptive arms of the immune system are affected, of which the underlying mechanisms remain to be unraveled. At least part of the reduced immune function in vivo can be explained by a substantial drop in numbers of circulating leukocytes (leucopenia) during torpor, as demonstrated for all species of hibernating animals studied thus far [7, 9–14]. Previously, we demonstrated that low body temperature affects the number of circulating lymphocytes during torpor through a reduced plasma level of S1P, resulting in impaired lymphocyte egress from lymphoid tissues [10].

The explanation for the decrease in number of circulating neutrophils during torpor is less clear. One mechanism could be retention of neutrophils in lungs, as numbers of neutrophils in lungs increase during torpor in hibernating hedgehogs (*Erinaceus europaeus*) [15]. Studies with mongrel dogs (*Canis familiaris*) also revealed that hypothermia induced a reversible leucopenia as a result of adherence of cells to the endothelium of mesenteric venules [16]. However, whether this mechanism leads to a reduced number of circulating neutrophils during torpor is not known. Therefore, in the present study, we determined whether transient neutropenia during torpor (and hypothermia) is a result of temporary attachment to vessel walls or whether alternative mechanisms are involved. We performed experiments in naturally hibernating and in nonhibernating (forced hypothermic) animals to better identify the underlying mechanisms that lead to transient neutropenia during torpor.

1. Correspondence: Dept. of Clinical Pharmacology/FB20, University Medical Center Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: h.r.bouma@umcg.nl

Abbreviations: L:D=light:dark, S1P=sphingosine-1-phosphate

MATERIALS AND METHODS

Induction of deep torpor in Syrian hamsters

Male and female Syrian hamsters (*Mesocricetus auratus*) were bred in the animal facilities of the University of Groningen (The Netherlands). Animals were housed at an ambient temperature of $21 \pm 1°C$ and summer photoperiod (L:D cycle, 14:10 h). Torpor was induced by first shortening the L:D cycle to 8:16 h for ~10 weeks, followed by housing at continuous dim light (<5 Lux) at an ambient temperature of 5°C; animals at this stage that did not show signs of torpor behavior were called "winter euthermic". During hibernation, Syrian hamsters exhibit deep torpor bouts of 3–6 days, alternating with arousal periods of ~23 h [17]. Torpor-arousal patterns were monitored by movement detectors connected to a computer. Hamsters were killed during winter euthermia, torpor, or during interbout arousal (referred to as "arousal"). Experiments with Syrian hamsters were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen.

Induction of daily torpor in Djungarian hamsters

Male and female Djungarian hamsters were bred in the animal facilities of the Rowett Institute for Nutrition and Health at the University of Aberdeen (UK). All animal work was licensed under the Animals (Scientific Procedures) Act of 1986. Prior to the experiments, animals were housed at an ambient temperature of $21 \pm 1°C$ and a summer-like photoperiod (L:D, 16:8). Daily torpor was induced by shortening the L:D cycle to L:D 8:16 for ~14 weeks at an ambient temperature of 21°C. Visual inspection of torpid behavior (absence of activity and typical torpor posture) was carried out in the middle of the light phase, which is the usual torpor time for Djungarian hamsters. Animals were killed during winter euthermia (time-matched euthermic control for torpid animals), torpor, or arousal (±12 h following a bout of daily torpor).

Rats

Male Wistar rats (*Rattus norvegicus*), weighing 350–400 g, were obtained from Harlan Netherlands B.V. (The Netherlands). Experiments on rats were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen.

Forced hypothermia

Summer euthermic Syrian hamsters housed at a L:D cycle of 14:10 h and Wistar rats housed at a L:D cycle of 12:12 h were anesthetized by i.p. injection of 200 mg/kg ketamine and 2 mg/kg diazepam. After induction of anesthesia, a catheter was inserted into the jugular vein for blood sampling. Rectal temperature and heart rate (electrocardiography) were monitored continuously (Cardiocap S/5; Datex Ohmeda, GE Healthcare, Bloomfield, CT, USA). Following cannulation, an initial blood draw, and installation of the monitoring devices, animals were cooled by applying ice-cold water on the fur until they reached a body temperature of 10–15°C. These animals could be rewarmed without ventilatory support. Rats had to be ventilated throughout the procedure to allow safe cooling to a body temperature of ~15°C and subsequent rewarming. Therefore, following intubation, rats were ventilated mechanically (Amsterdam Infant Ventilator; Hoek Loos, The Netherlands). The tidal volume was set to achieve normocapnia, as verified by capnography and arterial blood gas analysis, with O_2:air (1:2) at a ventilation rate of 50 min^{-1} (0.5 s inspiration time). After reaching the desired low body temperature, animals were rewarmed immediately using a water-based heating mattress. Cooling and rewarming rates were ~1°C/3 min. Blood samples were taken at several body temperatures during cooling—when animals reached the desired low body temperature, during rewarming, and at least 10 min after a euthermic temperature (>35°C) was reached. In another experiment, summer euthermic Syrian hamsters were pretreated 4 h preceding forced hypothermia with 10 mg/kg dexamethasone or saline i.p., and a baseline blood sample was drawn by orbital puncture. They were then cannulated, cooled, and rewarmed as described

above, and blood samples were drawn at the induction of anesthesia upon reaching the desired low body temperature and following rewarming. In all experiments, blood (~200 μl) was collected via the jugular vein catheter in EDTA-coated cups (MiniCollect K3 EDTA; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) for automated hematological analysis using a XE-2100 (Sysmex, Etten-Leur, The Netherlands) [18, 19].

Splenectomy

Splenectomies were performed on summer euthermic and torpid Syrian hamsters. After induction of anesthesia with isoflurane (2–2.5% in O_2), flunixin-meglumin was given s.c. (4 mg/kg) for analgesia. A small, abdominal, midline incision was made, the arteries and veins toward and from the spleen were ligated, and the spleen was resected, followed by wound closure. Splenectomized summer euthermic animals were allowed to recover at ~20°C (L:D cycle, 14 h:10 h) for at least 1 week before induction of torpor. They were killed during their third torpor bout, which was 60.3 ± 8.1 days following splenectomy. Torpid animals were splenectomized during their third torpor bout. These animals were kept at <10°C using ice packs. Animals recovered in a climate-controlled room at an ambient temperature of ~5°C (continuous dim light) and were sacrificed directly after reaching euthermia (>33°C) following arousal. The perioperative handling of animals induced the onset of arousal, which started immediately, followed by weaning from anesthesia.

Sacrification and sample collection

Syrian hamsters were killed by i.p. injection of an overdose of pentobarbital, followed by decapitation. Djungarian hamsters were killed with CO_2 and then decapitated. Rats were killed after the last blood sample was drawn by decapitation, while anesthetized. After euthanasia (but before decapitation), body temperature was measured rectally, and blood (~200 μl) was collected via cardiac puncture in EDTA-coated cups (MiniCollect K3 EDTA; Greiner Bio-One). This blood sample was analyzed with an automated hematology analyzer (XE-2100; Sysmex) [18, 19]. Differential leukocyte counts were validated manually using Wright-Giemsa-stained blood smears. Spleens from torpid and aroused Syrian hamsters were removed, snap-frozen in liquid nitrogen, and stored at −80°C.

Statistical analysis and data presentation

Data are presented as mean $\pm$ SEM. Group comparisons were performed using a two-tailed, independent-sample Student's *t*-test in the case of two groups. In the case of more than two groups, a one-way ANOVA with post hoc Tukey on normally distributed variables (based on homogeneity of variances) or a Kruskal-Wallis test, followed by a Mann-Whitney U-test in the case of non-normally distributed variables, was performed. Statistical differences were calculated using SPSS 20.0, where $P < 0.05$ was considered significantly different.

RESULTS

Deep torpor and daily torpor are associated with a reduced number of circulating leukocytes

Body temperatures of Syrian hamsters during winter euthermia and deep torpor were $34.0 \pm 0.5°C$ (*n*=8) and $6.2 \pm 0.1°C$ (*n*=8), respectively (*P*<0.01). Numbers of circulating leukocytes were 2.92 ± 0.71 ($\times 10^6$/ml) during winter euthermia and 0.12 ± 0.04 ($\times 10^6$/ml) in deep torpor (*P*<0.01). After 5–6 days of torpor, animals aroused spontaneously, and the number of leukocytes increased rapidly to 2.72 ± 0.34 ($\times 10^6$/ml; *n*=8; *P*<0.01), i.e., to the same level as seen in winter euthermic animals. The numbers of erythrocytes in the blood were similar in winter euthermic hamsters

($8.96\pm0.42\times10^9$/ml), torpid hamsters ($9.05\pm0.26\times10^9$/ml), and aroused animals ($7.71\pm0.49\times10^9$/ml).

Body temperatures of Djungarian hamsters during winter euthermia and daily torpor were $35.0 \pm 0.2°C$ (n=6) and $25.2 \pm 1.3°C$ (n=8), respectively (P<0.01). The number of circulating leukocytes declined from 9.50 ± 0.85 ($\times10^6$/ml) in winter euthermia to 5.02 ± 0.41 ($\times10^6$/ml) in torpor (P<0.01) and then rose again to winter euthermic levels 8.22 ± 0.96 ($\times10^6$/ml) upon arousal (n=5; P<0.01). Erythrocyte counts in Djungarian hamsters remained stable during hibernation: 9.45 ± 0.33 ($\times10^9$/ml) in euthermia, 8.80 ± 0.31 ($\times10^9$/ml) during daily torpor, and 9.67 ± 0.15 ($\times10^9$/ml) upon arousal. These results suggest that leucopenia is not restricted to deep torpor but also occurs in animals exhibiting daily torpor.

Deep and daily torpor are associated with neutropenia

Leucopenia during torpor is a result of a decrease in numbers of all major classes of leukocytes, including neutrophils, lymphocytes, and monocytes [7, 9–14]. In Syrian hamsters, the number of circulating neutrophils decreases during entry into deep torpor as body temperature falls, and numbers are correlated strongly with the body temperature (P=0.679; n=79; P<0.01). The number of circulating neutrophils is 0.92 ± 0.31 ($\times10^6$/ml; **Fig. 1A**) during winter euthermia and 0.02 ± 0.00 ($\times10^6$/ml; P<0.05; Fig. 1B) during torpor. Numbers of circulating neutrophils are restored to 1.50 ± 0.35 ($\times10^6$/ml; P<0.01; Fig. 1C) upon arousal. Interestingly, arousal from torpor is associated with a greater number of circulating neutrophils compared with animals that had not yet entered torpor (i.e., winter euthermic animals) or following rewarming from forced hypothermia (see below). During daily torpor in the Djungarian hamster, the number

of circulating neutrophils falls significantly during torpor compared with winter euthermic animals and is followed by full restoration upon arousal (Fig. 1D).

Forced hypothermia induces neutropenia in hamsters

The effect of low body temperature on the number of circulating neutrophils was demonstrated further by cooling anesthetized summer euthermic Syrian hamsters ("forced hypothermia"). Cooling to a body temperature of $9.1 \pm 0.8°C$ induces a decrease in the number of circulating neutrophils, as observed in deep torpor: from 0.54 ± 0.09 ($\times10^6$/ml; n=5) to 0.02 ± 0.01 ($\times10^6$/ml; n=5; P<0.01; Fig. 1B and C). Thus, this finding indicates that simply lowering the body temperature leads to the induction of neutropenia.

The spleen is not the only organ involved in induction or restoration of neutropenia

To evaluate a potential role of spleen in storage of circulating neutrophils during torpor, we examined the effect of splenectomy on the numbers of circulating neutrophils. Syrian hamsters were splenectomized before entering hibernation or during torpor. Removal of the spleen before hibernation did not affect the number of circulating neutrophils during torpor; i.e., neutrophil numbers in splenectomized torpid hamsters are similar to those in nonsplenectomized animals (Fig. 1E). In addition, splenectomy during torpor did not prevent the rise in the number of circulating neutrophils upon arousal (Fig. 1F). However, following removal of the spleen during torpor, the number of circulating neutrophils was significantly higher in aroused animals that were splenectomized during torpor compared with nonsplenectomized animals (P<0.05;
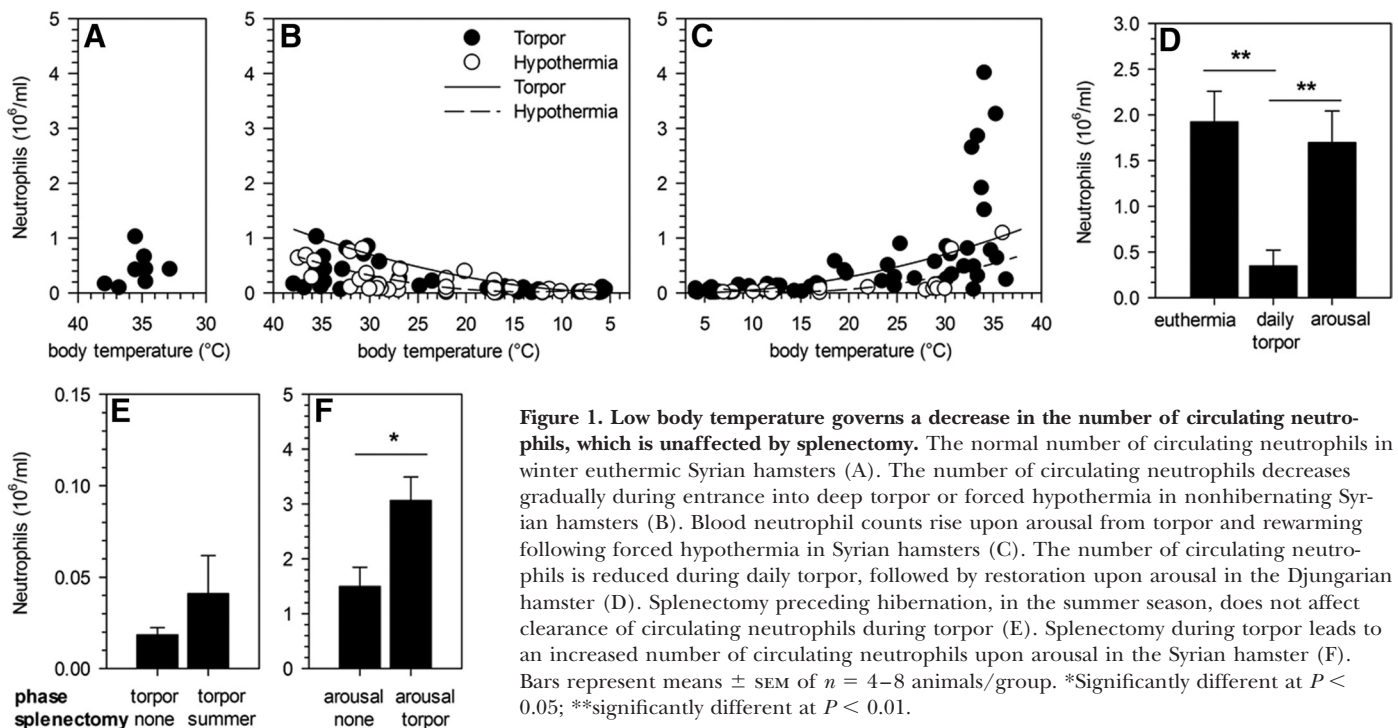

Figure 1. Low body temperature governs a decrease in the number of circulating neutrophils, which is unaffected by splenectomy. The normal number of circulating neutrophils in winter euthermic Syrian hamsters (A). The number of circulating neutrophils decreases gradually during entrance into deep torpor or forced hypothermia in nonhibernating Syrian hamsters (B). Blood neutrophil counts rise upon arousal from torpor and rewarming following forced hypothermia in Syrian hamsters (C). The number of circulating neutrophils is reduced during daily torpor, followed by restoration upon arousal in the Djungarian hamster (D). Splenectomy preceding hibernation, in the summer season, does not affect clearance of circulating neutrophils during torpor (E). Splenectomy during torpor leads to an increased number of circulating neutrophils upon arousal in the Syrian hamster (F). Bars represent means ± SEM of $n = 4$–8 animals/group. *Significantly different at $P < 0.05$; **significantly different at $P < 0.01$.

Fig. 1F). Thus, the spleen is not essential for retention or storage of large numbers of neutrophils during torpor.

The percentage of circulating, immature granulocytes is not altered during hibernation

We speculate that if massive apoptosis of neutrophils occurs during torpor, then substantial release of (newly produced) neutrophils from the bone marrow should occur upon arousal to restore the number of circulating cells. To obtain information about the number of newly produced neutrophils, we measured the number of immature granulocytes in the Syrian hamster. Numbers of immature granulocytes are very low in summer, winter euthermic, and hibernating (torpid and aroused) animals (detection limit of 0.001×10^6 cells/ml). Although this low number hampers exact quantification of immature granulocytes, it does rule out significant release of immature (neutrophilic) granulocytes from the bone marrow upon arousal. Hence, massive apoptosis of neutrophils seems unlikely to be the sole explanation for neutropenia during torpor.

Low body temperature also drives neutropenia in nonhibernating species (rats)

We found that the number of circulating neutrophils is reduced during deep and daily torpor in hibernating hamsters, as well as during forced hypothermia in nonhibernating hamsters. To assess whether the induction of neutropenia by low body temperature is a general mechanism not restricted to hibernating species, we induced forced hypothermia in rats (*R. norvegicus*), a nonhibernating species. Lowering the body temperature results in a concomitant decrease in the number of circulating leukocytes, which is a result of reduced numbers of neutrophils, lymphocytes, monocytes, and eosinophils but not basophils (**Table 1**). However, the number of circulating erythrocytes remains stable during forced hypothermia. In contrast to hamsters, rats have a higher number of circulating, immature granulocytes. Cooling the rats reduced the number

of immature granulocytes to values below the detection threshold, although not significantly different from the number before cooling (i.e., $<0.001 \times 10^6$/ml; Table 1). Following rewarming, the number of immature granulocytes rose to values that were not significantly different from precooling levels (Table 1). The absence of a significant rise in the number of immature granulocytes following hypothermia compared with baseline values suggests that the restoration of circulating neutrophils upon rewarming is likely not a result of release of immature cells from the bone marrow.

Pretreatment with dexamethasone prevents neutropenia during forced hypothermia

As apoptosis and reduced release from the bone marrow are unlikely explanations for the kinetics of neutrophils during torpor and arousal, we explored whether temporary retention of cells might lead to neutropenia during torpor. Although the spleen is not involved in the induction of neutropenia during torpor, an alternative explanation for transient neutropenia during torpor and hypothermia is temporary retention of neutrophils in organs as a result of margination of cells to vessel walls. Thus, we injected summer euthermic Syrian hamsters prior to forced hypothermia with 10 mg/kg dexamethasone i.p. to inhibit margination of neutrophils [20–22]. As shown in **Fig. 2A** and **B**, dexamethasone pretreatment did not affect the induction of hypothermia or the associated drop in heart rate. However, it abolished the neutropenia that normally occurs during hypothermia (Fig. 2B) but had no effect on the number of circulating erythrocytes (Fig. 2C). As dexamethasone can also stimulate release of neutrophils from the bone marrow, in addition to demargination of cells [20–22], we counted the number of immature granulocytes in blood. The number of circulating, immature granulocytes was comparable with that during hibernation, around the detection limit of 0.001×10^6 cells/ml. These results support the hypothesis that low body temperature governs neutropenia by margination.

TABLE 1. Forced Hypothermia in a Nonhibernating Animal (the Rat) Leads to a Reduced Number of Circulating Neutrophils, Lymphocytes, and Monocytes

	Wistar rat		
	Euthermia (n=5)	Hypothermia (n=5)	Rewarming (n=5)
Body temperature	37.0 ± 0.0^{HT}	$15.4 \pm 0.4^{EU,REW}$	37.0 ± 0.0^{HT}
Erythrocytes	7.81 ± 0.20	6.84 ± 0.72	7.48 ± 0.49
Leukocytes	8.82 ± 1.07^{HT}	$0.57 \pm 0.20^{EU,REW}$	7.68 ± 1.53^{HT}
Neutrophils	1.63 ± 0.49^{HT}	$0.15 \pm 0.04^{EU,REW}$	2.59 ± 0.75^{HT}
Lymphocytes	6.76 ± 0.96^{HT}	$0.39 \pm 0.19^{EU,REW}$	4.79 ± 1.37^{HT}
Monocytes	0.34 ± 0.08^{HT}	0.03 ± 0.01^{EU}	0.21 ± 0.11
Eosinophils	0.08 ± 0.03	0.00 ± 0.00	0.08 ± 0.03
Basophils	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
Immature granulocytes	0.012 ± 0.005	0.00 ± 0.00	0.012 ± 0.005

Table shows the body temperature (°C), the number of circulating erythrocytes ($\times 10^9$/ml) and leukocytes ($\times 10^6$/ml), and differentiated leukocyte counts ($\times 10^6$/ml) during euthermia (before experiment) and forced hypothermia and following full rewarming in the Wistar rat. Shown are means ± [scap]sem of $n = 5$ animals/group. Significant differences ($P<0.05$) among groups are indicated by the superscripts (HT, hypothermia; EU, euthermia; REW, rewarming).
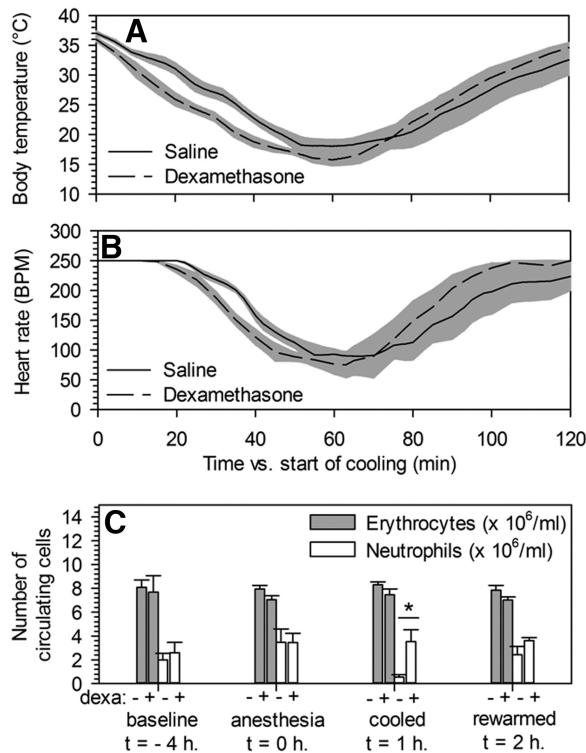
Figure 2. Neutropenia induced by low body temperature in a hibernator is a result of margination of neutrophils. Forced hypothermia of anesthetized summer active Syrian hamsters leads to a profound reduction in body temperature that was unaffected by dexamethasone pretreatment i.p. (A). Lowering of body temperature is associated with a severe drop in heart rate, both in saline as well as in dexamethasone-pretreated hamsters (B). Although hypothermia did not affect erythrocyte counts, the number of neutrophils was transiently reduced by hypothermia, which was precluded by pretreatment with dexamethasone ($P<0.05$; C). BPM, Beats/min; dexa, dexamethasone (10 mg/kg)-pretreated animals. Graphs represent means ± SEM (shown as gray-shaded areas or by error bars, respectively) of $n = 4$ (saline) and $n = 6$ (dexamethasone) animals/group. *Significantly different at $P < 0.05$.

DISCUSSION

In all hibernating animals studied so far, the number of circulating leukocytes decreases after entrance into torpor, followed by rapid restoration upon arousal [8]. Several studies demonstrated that leucopenia during torpor is a result of a reduction in the number of circulating lymphocytes, neutrophils, and monocytes [9–12, 14, 23]. Previously, we showed that the drop in lymphocytes during hibernation is driven by low body temperature that directly results in lower S1P levels in the blood [10]. In the current study, we demonstrate that the reduction in circulating neutrophils is also driven by the decrease in body temperature during torpor, as neutropenia occurs in deep hibernators, as well as during daily torpor in the Djungarian hamster. Moreover, neutropenia is also induced by forced hypothermia in summer euthermic Syrian hamsters and in rats, a nonhibernating species.

The mechanism underlying the reduction of neutrophils in blood might be explained by lower production of neutrophils,

increased apoptosis, and/or temporary retention of cells during torpor. The half-life of neutrophils is estimated to be 14 h (as measured in mice) [24, 25]. Therefore, we speculate that if a reduced release from the bone marrow is the sole explanation for neutropenia during torpor, a period of 3–5 days is required to lead to the low number of circulating neutrophils, as observed during torpor. However, the number of circulating leukocytes correlates closely with the reduction in body temperature. Furthermore, ~95% of the leukocytes are cleared from the circulation as soon as the animals approach their torpid body temperature, which is well within 14 h. Thus, it is unlikely that a lower production rate of neutrophils by the bone marrow contributes significantly to the drop in cell numbers observed during torpor. Neutropenia during torpor is also not explained by massive apoptosis of neutrophils, as there is no difference in the number of immature, circulating neutrophils during arousal from deep torpor or rewarming from forced hypothermia in Syrian hamsters or rats, and the fraction of immature granulocytes remains very low. Thus, a reduced production of cells and/or apoptosis cannot explain the rapid induction of leucopenia during torpor or forced hypothermia. Furthermore, restoration of circulating neutrophils upon arousal or following rewarming from hypothermia does not appear to be a result of rapid regeneration in the bone marrow.

An alternative explanation is that neutrophils are temporarily retained at certain sites during torpid periods and are released again during arousal. Villalobos et al. [26] demonstrated a comparable phenomenon to occur in deep hypothermic dogs, leading to leucopenia and a reduction in the number of platelets. In line with our results, rewarming of the dogs induced restoration of the number of circulating leukocytes and platelets. Comparison of the number of circulating platelets in blood samples derived from aorta, upper and lower vena cava, and capillaries (of the tongue) did not reveal specific retention in the arterial, capillary, or venous vascular system. Splenectomizing and hepatectomizing dogs, prior to hypothermia, reduced the extent of trombopenia. However, neither splenectomy nor hepatectomy or splenectomy combined with hepatectomy precluded trombopenia induced by hypothermia. Thus, hypothermia leads to reversible clearance of circulating leukocytes and platelets, which does not seem to be confined to arteries, capillaries, or veins and in which the spleen and liver do not play a key role.

Our findings also indicate that the spleen does not play a significant role in storage of neutrophils during torpor. Splenectomy, preceding the hibernation season, does not affect the reduction in the number of circulating neutrophils nor did splenectomy of torpid animals prevent restoration of normal numbers of circulating neutrophils during subsequent arousal to euthermia. Although these results suggest that the spleen does not play an essential role, they do not completely rule out a role in the induction or restoration of neutropenia during hibernation. In contrast, our data support the hypothesis that neutrophils stop circulating during torpor as a result of reversible adherence to endothelial cells of blood vessel walls (margination), as neutropenia induced by low body temperature was fully blocked by dexamethasone pretreatment in our model of forced hypothermia. Margination depends on the

interaction between leukocytes and endothelial cells as a result of expression of integrins, selectins, and adhesion molecules and detaching forces, such as shear forces, because of blood flow velocity [27, 28]. Studies in rabbits show that under normal circumstances, a substantial number of neutrophils are marginated; the marginated fraction can be as high as ~61% of the number of circulating neutrophils [20]. Dexamethasone reduces margination of neutrophils through mechanisms that are not fully understood yet [20–22]. Possibly, reduced expression of L-selectin and CD18 on neutrophils [29, 30] and endothelial-leukocyte adhesion molecule-1 and ICAM-1 on endothelial cells [31], observed after dexamethasone treatment of cells, plays a role in demargination of neutrophils. In addition, glucocorticoids can increase the half-life of neutrophils and stimulate release from the bone marrow through G-CSF [21, 22]. The number of circulating, immature granulocytes remains low after injection of dexamethasone, which suggests that the effect of dexamethasone on hypothermia-induced neutropenia is not a result of stimulation of bone marrow release. Thus, low body temperature governs neutropenia by margination, which likely explains the neutropenia associated with torpor.

The mechanism by which low body temperature induces margination is not yet fully understood. In general, margination of neutrophils is promoted by adhering forces, such as the expression of adhesion molecules and integrins, and is reduced by detaching forces, such as increased shear stress by blood flow. Direct effects of lowered body temperature are unlikely to cause neutropenia by means of margination, as low temperature reduces the expression of ICAM-1 in lung in vivo [32] and E-selectin on endothelial cells in vitro [33, 34]. However, these effects might be compensated for by indirect effects of low body temperature in vivo, such as a reduced cardiac output and the subsequent drop in blood flow velocity that might increase leukocyte-endothelium adhesion. Our data support the hypothesis that lowered body temperature induces margination, an effect that is not restricted to hibernating species, as we also observed this phenomenon in the rat. However, the existence of additional effects, specific for hibernators, cannot be excluded. Yasuma et al. [35] showed that incubation of rat endothelial cells at 37°C in vitro with plasma from hibernating but not from euthermic 13-lined ground squirrels leads to up-regulation of ICAM-1 expression and increased monocyte adhesion to these cells. These findings suggest that in addition to lowered body temperature, temperature-independent effects of torpor might affect the expression of adhesion molecules, which could potentially promote margination of neutrophils during torpor in vivo. In summary, adherence of neutrophils to vessel walls increases when body temperature falls. This finding may explain the reduced number of circulating cells during deep torpor, daily torpor, and forced hypothermia.

Neutropenia during torpor may also contribute to the diminished function of the innate-immune system during torpor. A reduced innate-immune function has been demonstrated by i.p. injection of LPS during deep torpor, which does not lead to a febrile response, as long as the animals remain torpid. However, injection of LPS during arousal induced a febrile response and prolonged the duration of arousal [36]. Possibly, reversible reduction of the innate-immune system by margination of cells allows conservation of energy while maintaining the potential of rapid reactivation in case of infection. The understanding of the mechanisms involved in the reduced function of the immune system may be relevant to the pathophysiological consequences of therapeutic hypothermia [37]. Although hypothermia is used clinically to stabilize trauma patients and during major surgery to suppress metabolic activity and limit organ injury during periods of low oxygen supply, it is associated with the occurrence of serious side-effects, such as fatal thrombosis [38–40], neurogical deficits [41], renal injury [42], and increased blood loss [43]. Organ injury might be exaggerated by inflammation with influx of neutrophils, in which adhesion (margination) of neutrophils is an important, initial step. Indeed, experimental blockade of margination reduces the extent of pulmonary injury following normothermic cardiopulmonary bypass in a sheep model [44]. Interestingly, hypothermia in humans leads to the induction of a reversible leucopenia, comparable with hibernating animals and hypothermic rats [45]. Possibly, the increased resistance to ischemia/reperfusion and hypothermia of hibernating animals [46–53] prevents the occurrence of cellular injury and the subsequent onset of an inflammatory response, as might occur in humans during treatment with cardiopulmonary bypass [42, 51, 54–56]. Thus, margination of neutrophils induced by low body temperature might be involved in the etiology of organ injury following hypothermia in humans.

Conclusion

Low body temperature induces a decrease in the number of circulating neutrophils, which is not restricted to hibernating species. Our data support the hypothesis that neutropenia, as a result of low body temperature, is caused by reversible margination of cells rather than by apoptosis of cells, a reduced release from the bone marrow, or retention in the spleen. Despite the fact that hibernators regularly cycle through periods with an extremely reduced body temperature, followed by rapid rewarming, no gross signs of organ injury are evident. Translating the underlying mechanisms that govern increased resistance to hypothermic injury may therefore be of major clinical relevance.

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