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Short communication

Blood cell dynamics during hibernation in the European Ground Squirrel

H.R. Bouma[a,b,*], A.M. Strijkstra[b], A.S. Boerema[b], L.E. Deelman[a], A.H. Epema[c,d], R.A. Hut[b], F.G.M. Kroese[d], R.H. Henning[a]

[a] Department of Clinical Pharmacology, Groningen University Institute for Drug Exploration (GUIDE), University Medical Center Groningen, The Netherlands
[b] Department of Chronobiology, University of Groningen, The Netherlands
[c] Department of Anesthesiology, University Medical Center Groningen, The Netherlands
[d] Department of Cell Biology/Section Immunology, University of Groningen, The Netherlands

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ABSTRACT

Hibernation is a unique natural model to study large and specific modulation in numbers of leukocytes and thrombocytes, with potential relevance for medical application. Hibernating animals cycle through cold (torpor) and warm (arousal) phases. Previous research demonstrated clearance of leukocytes and thrombocytes from the circulation during torpor, but did not provide information regarding the timing during torpor or the subtype of leukocytes affected. To study the influence of torpor-bout duration on clearance of circulating cells, we measured blood cell dynamics in the European Ground Squirrel. Numbers of leukocytes and thrombocytes decreased within 24 h of torpor by 90% and remained unchanged during the remainder of the torpor-bout. Differential counts demonstrated that granulocytes, lymphocytes and monocytes are all affected by torpor. Although a decreased production might explain the reduced number of thrombocytes, granulocytes and monocytes, this cannot explain the observed lymphopenia since lymphocytes have a much lower turnover rate than thrombocytes, granulocytes and monocytes. In conclusion, although underlying biochemical signaling pathways need to be unraveled, our data show that the leukocyte count drops dramatically after entrance into torpor and that euthermic cell counts are restored within 1.5 h after onset of arousal, even before body temperature is fully normalized.

1. Introduction

Hibernation is a behavior conserved throughout the animal kingdom that results in energy conservation and allows animals to survive under harsh conditions. In the winter season, some small rodents regularly enter a state of metabolic suppression, which results in a drop of body temperature close to environmental temperature. Such phases (known as 'torpor') are regularly interspersed with shorter phases of full rewarming ('arousal') in which body temperature restores to normal euthermic values within a few hours (Hut et al., 2002). Depending on the species and temperature, a torpor-bout may last from 4 to 30 days. Torpor-bouts are characterized by major changes of physiological parameters, including a substantial reduction of heart rate, ventilation rate, blood pressure and urinary output (Lyman and Chatfield, 1955). In addition, other physiological changes, such as a seeming resistance to ischemic states and an improved anti-oxidant defense, are believed to protect animals from notable organ damage during the repetitive transitions from torpor to aroused

* Corresponding author at: Department of Clinical Pharmacology/FB20, University Medical Center Groningen, Antonius Deusinglaan 1/FB20, 9713 AV Groningen, The Netherlands. Tel.: +31 50 363 2810, fax: +31 50 363 2812.
E-mail address: h.r.bouma@med.umcg.nl (H.R. Bouma).

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phases and vice versa (Carey et al., 2000; Kurtz et al., 2006).

Adaptations in the immune system may serve to limit organ damage further. Indeed, when animals are injected with endotoxin during torpor, no fever or arousal is induced, but fever can be observed during the next arousal (Prendergast et al., 2002). Isolated macrophages from animals in the pre-hibernation period or during torpor in the hibernation period show a reduced basal TNF-α level than macrophages from aroused or summer euthermic animals (Novoselova et al., 2000). One of the most striking adaptations during the torpid phase consists of a dramatic decrease in circulating numbers of leukocytes and thrombocytes, both of which restore after hibernation (Frerichs et al., 1994; Lechler and Penick, 1963; Lyman and Chatfield, 1955; Reddick et al., 1973; Reznik et al., 1975; Spurrier and Dawe, 1973). These phenomena during torpor may result from lowered body temperature per se, as hypothermia (*in vitro*) has been reported to irreversibly activate thrombocytes and leads to the formation of thrombocyte/leukocyte-aggregates (Straub et al., 2005, 2007; Xavier et al., 2007). Aggregate-formation might give rise to a cold-induced leuko- and thrombopenia, which is also observed in humans (Shenaq et al., 1986). Although formation of aggregates explains the hypothermia-induced leuko- and thrombopenia very well, one does not expect this mechanism to be fully reversible upon rewarming. Our study was designed to determine blood cell changes at more defined time-points in the torpor–arousal cycle than had been studied previously. We assessed whether the hibernation-associated decrease in leuko- and thrombocytes is fully reversible upon arousal, and whether different subtypes of leukocytes behave differently during hibernation. To this end, we performed full blood counts of circulating blood cells in European Ground Squirrels at several time-points during different phases of hibernation.

2. Materials and methods

2.1. Animals

European Ground Squirrels (*Spermophilus citellus*, $n = 30$) were acquired and housed as described previously (Henning et al., 2002). Briefly, animals were kept in lucite cages with a nest box attached. Rabbit breeding chow (Teurlings, Waalwijk, The Netherlands) and water were provided ad libitum. To induce torpor, ambient temperature was gradually lowered from 20 °C to 5 °C and light:dark-patterns were shortened from 12 h:12 h light:dark, to continuous dim light (<1 lx). To assess the individual torpor or euthermic states, nest box temperatures and activity were measured every minute using a computer-based recording system. The experiments were approved by the Animal Experiments Committee of the University of Groningen (DEC#BG02198).

To assess the effect of torpor (and torpor-bout duration) on levels of circulating blood cells, the temperature of the climate-controlled rooms was set to 5 °C. Once the animals demonstrated a stable hibernation pattern, torpid animals were sacrificed after 1 day ($n = 5$), 4 days ($n = 5$) or 1 week of torpor ($n = 5$). To precisely determine the start of arousal, an arousal was induced at the end of a natural torpor-bout by handling the animals. Aroused animals were sacrificed 1.5 h ($n = 5$; early arousal) and 8 h ($n = 6$; late arousal) after the start of arousal. Summer euthermic animals served as controls ($n = 4$).

2.2. Sample collection and analysis

After terminal anesthesia by an overdose of pentobarbital, 250 μL of blood was collected in EDTA-coated cups (Greiner mini-collect ref. no. 450476) for analysis within 5 h on the Sysmex XE-2100, an automated hematology analyzer (Briggs et al., 2000; Ruzicka et al., 2001). In addition, morphological thresholds for subtype identification were verified from scatter plots obtained from the hematology analyzer and Giemsa-stained blood smears were analyzed to validate the automated cell counts. Previous studies have demonstrated successful analysis of blood derived from small mammals using the Sysmex system (Lilliehöök and Tvedten, 2009; Kabata et al., 1991). Spleens from a randomly selected subset of animals were fixed in 4% paraformaldehyde, embedded in paraffin and stained using hematoxylin/eosin. Image Pro Plus 6 was used to analyze microscopic images and calculate white pulp size that was expressed as % of total spleen tissue area.

2.3. Statistical analysis

Significant differences ($p < 0.05$) were calculated using ANOVA and post hoc LSD/Tamhane or a Two-Tailed Student's *T*-test in the case where less than three groups were to be compared (SPSS 16.0).

3. Results and discussion

3.1. Body temperature and hydration status

Summer euthermic animals had body temperatures of 35.7 ± 0.7 °C, which decreased rapidly on entrance of torpor to 10.2 ± 2.0 °C after 24 h and further decreased to 7.6 ± 0.2 °C and 8.2 ± 0.3 °C after 4 and 7 days of torpor, respectively ($p < 0.01$; Fig. 1, inset). Body temperature increased rapidly again upon arousal towards 30.9 ± 1.8 °C and 34.5 ± 0.3 °C at 1.5 h and 8 h after onset of arousal. The hematocrit and erythrocyte count were measured to estimate the influence of hydration status of the animals (and fluid shifts) on numbers of circulating cells. No differences were found in hematocrit, which was 0.47 ± 0.01 L/L in summer euthermic animals, 0.50 ± 0.01 L/L during torpor and 0.47 ± 0.01 L/L during arousal. Erythrocyte counts also remained unaffected throughout hibernation: the erythrocyte count in summer euthermic animals was $8.3 \pm 0.1 \times 10^{12}$/L, $8.7 \pm 0.3 \times 10^{12}$/L during torpor and $8.1 \pm 0.3 \times 10^{12}$/L during arousal.

3.2. Thrombocyte counts are reduced during torpor

During the first 24 h of torpor, thrombocyte counts decreased extremely, but remained unchanged during the remainder of the torpor-bout. Although thrombocyte counts increased rapidly within 1.5 h after onset of

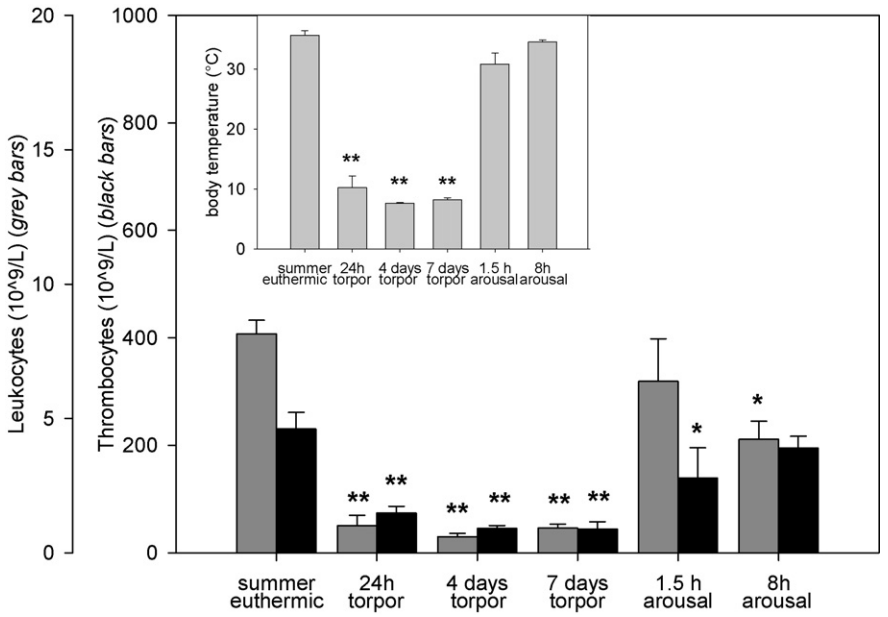

Fig. 1. Changes in body temperature and numbers of circulating leukocytes (*grey bars*) and thrombocytes (*black bars*) in the summer and during hibernation in the European Ground Squirrel.
Shown in the figure are rectal body temperatures (°C) (inset), leukocyte (grey bars) and thrombocyte (black bars) counts (10^9/L) in animals at different time-points in the summer and during hibernation. Samples were drawn by cardiac punction after 24 h (TS; $n = 5$), 4 days (TM; $n = 5$), and 7 days (TL; $n = 5$) of torpor and 1.5 h (AS; $n = 5$) and 8 h after the start of arousal (AL; $n = 6$). Blood from summer euthermic animals (EU; $n = 4$) served as control. Significant differences were calculated using ANOVA and post hoc LSD/Tamhane tests. Data are shown as mean ± Standard Error of the Mean (SEM). */**$p < 0.05/0.01$ compared to summer euthermic animals (EU).

arousal, they were still significantly different from those found in summer euthermic animals. However, 8 h after onset of arousal the thrombocyte count was not significantly different from summer euthermic counts (Fig. 1). The increase in circulating thrombocytes during arousal might be due to rapid release from the bone marrow. Previous work has shown that bone marrow from hibernating Ground Squirrels contains almost twice as much megakaryocytes as bone marrow from non-hibernating animals (Szilagyi and Senturia, 1972). Thrombocytopenia triggers megakaryocytes to respond by increasing ploidy of cells, followed by release of newly formed with a relative high mean thrombocyte volume (Corash et al., 1987). In the squirrels we found a significantly higher mean thrombocyte volume (MPV) in winter-animals ($7.14 \pm 0.35 \times 10^9$/L during torpor and $6.91 \pm 0.91 \times 10^9$/L during arousal) compared to summer euthermic animals ($5.48 \pm 0.39 \times 10^9$/L; $p < 0.01$). These findings may suggest an increased production of thrombocytes during the winter season, probably to compensate for the increased loss of thrombocytes during torpor. The decrease in thrombocytes has been suggested to protect hibernators against *stasis thrombi* induced by the severely decreased body temperature, bradycardia and sluggish blood flow (Reddick et al., 1973), and possibly related to enhanced thrombocyte aggregation of blood at subnormal temperatures (Xavier et al., 2007).

3.3. Torpor induces leukocytopenia

Numbers of circulating leukocytes decreased in parallel with the extreme decrease in numbers of thrombocytes. The number of leukocytes decreased to values of less than 15% of euthermic values already after 24 h of torpor ($p < 0.01$) and remained stable but low over 4–7 days during the remainder of the torpor-bout. The leukocyte count increased rapidly within 1.5 h after onset of arousal to values not significantly different from those observed in summer euthermic animals. However, the number of circulating leukocytes 8 h after onset of arousal is significantly different from the number observed 24 h after onset of torpor and in summer euthermic animals ($p < 0.05$; Fig. 1). Since this time-point represents the middle of the interbout-arousal which lasts about 16 h, these data suggest rather restoration of homeostasis in cell counts towards a winter-euthermic level than induction of leukocytopenia to prepare for the coming torpor-bout. Combined with intracellular changes leading to a reduced cytokine production by macrophages and lymphocytes (i.e. IFN-α, -γ and TNF-α) as previously observed (Novoselova et al., 2000; Kandefer-Szerszen, 1988), the extreme reduction in the number of circulating cells will seriously affect the capacity to induce an effective immune response.

To obtain more information about subtypes of leukocytes involved in the torpor-associated leukocytopenia, we determined granulocyte, lymphocyte and monocyte counts during hibernation (Table 1). As can be seen from the table, the leukopenia affects both granulocytes (which are mainly neutrophilic granulocytes), monocytes and lymphocytes. Monocytes and granulocytes have a relative short life span, being much shorter than the length of a torpor-bout. The half-life of monocytes in mouse is estimated to be 22 h; granulocytes have a half-life of 13.7 h (Eash et al., 2009;

Table 1
Leukocyte subsets during hibernation in the European Ground Squirrel.

Leukocyte subtypes	Torpor (10^9/L)	Arousal (10^9/L)
Granulocytes	0.15 ± 0.04**	1.42 ± 0.47
Neutrophils	0.16 ± 0.03*	0.54 ± 0.25
Eosinophils	<0.01 ± 0.00	<0.01 ± 0.00
Basophils	0.01 ± 0.01*	0.87 ± 0.47
Lymphocytes	0.43 ± 0.07**	3.03 ± 0.93
Monocytes	0.04 ± 0.01**	0.21 ± 0.08

Shown in the table are numbers of circulating granulocytes (neutrophils, eosinophils and basophils), lymphocytes and monocytes (10^9/L) during torpor ($n = 13$) and arousal ($n = 7$). Significant differences were calculated using a Two-Tailed Student's *T*-test. Data are shown as mean ± Standard Error of the Mean (SEM).

* $p < 0.05$ compared to torpor.
** $p < 0.01$ compared to torpor.

Furth and Cohn, 1968). Probably, the observed decrease during torpor is due to a combination of decreased production secondary to hypothermia and the relatively short life span of the cells. During arousal, newly formed granulocytes can be released rapidly from the bone marrow. Although bone marrow from hibernating squirrels contains less cells than bone marrow from non-hibernating squirrels, it contains significantly more matured granulocytes during hibernation (Szilagyi and Senturia, 1972). Studies in mice demonstrated that under normal circumstances less than 2% of neutrophillic granulocytes are in the circulating pool, the remainder is in the bone marrow. Mice stimulated with granulocyte colony-stimulating factor (G-CSF) reach high production rates, thereby increasing their number of circulating granulocytes with 3×10^9/L in 1 h (Eash et al., 2009). This production rate would also be sufficient for a squirrel to increase its number of circulating granulocytes during arousal to euthermic levels (from $0.23 \pm 0.08 \times 10^9$/L during torpor to $1.40 \pm 0.46 \times 10^9$/L during arousal).

In contrast to the decreased number of circulating granulocytes, the lymphopenia is unlikely to be due to decreased production, since lymphocytes have a half-life of several months (Parretta et al., 2008; Sprent and Tough, 1994). To investigate whether lymphocytes go into apoptosis massively, we measured the white pulp size in the spleen in a subset of animals. Normally, lymphocytes circulate continuously through the spleen and in the case of massive apoptosis, white pulp size would decrease. White pulp size amounted 4.3 ± 1.4% in torpid animals ($n = 5$) and 2.9% and 4.3% during late arousal (One-Sample *T*-test: $p = 0.39$ and $p = 0.98$, respectively). Although major changes are seen in numbers of circulating lymphocytes, the size of white pulp in the spleen remains unaffected by hibernation. Thus, the lymphopenia during torpor is unlikely to be due to massive apoptosis of the cells. Further, numbers of circulating lymphocytes are rapidly restored during arousal. Lymphocytes cannot be produced as rapidly as granulocytes or thrombocytes. Studies in mice addressing the turnover rate of cells demonstrated that the time to label 50% of B cells with ^{3}HTdR or BrdU is in the order of weeks to months (Sprent, 1973, 1993). Proliferation rates of 0.2% per day are calculated for naïve T-cells, while memory cells proliferate at a speed of 1% per day (Parretta et al., 2008; Sprent and Tough, 1994; Tough and Sprent, 1994) in normal mice which have a thymus. However, seasonal thymic involution has been observed in hibernating animals during the winter (Galletti and Cavallari, 1972) and 5′-AMP released from brown adipose tissue in the winter is described to inhibit proliferation of lymphocytes (Atanassov et al., 1995). Extrathymic proliferation of naïve T cells in mice occurs at a very low rate: after 1 month only 5–10% of the naïve T-lymphocytes have incorporated BrdU, versus 70–80% of the memory T-lymphocytes (Sprent and Tough, 1994). Thus, the rapid recovery of normal lymphocyte counts within 1.5 h of euthermia seems incompatible with a hypothesis that cells go into apoptosis massively in torpor and are newly synthesized during arousal.

3.4. Potential retention mechanism of lymphocytes

Temporary storage or extravasation of cells might be caused by activation of adhesion molecules/homing receptors, an increased vascular permeability and/or decreased release from secondary lymphoid organs. Yasuma et al. demonstrated that rat cerebral microvascular endothelial cells cultured with serum from hibernating ground squirrels at 37 °C had significantly more activation of ICAM-1 than cells cultured with serum from non-hibernating squirrels (Yasuma et al., 1997). Interestingly, studies dealing with the effect of hypothermia on activation of ICAM-1, show a decreased activation when rats were treated with mild to moderate hypothermia after ischemia/reperfusion (Kira et al., 2005) or injection of LPS (Deng et al., 2003). Although serum from hibernating ground squirrels induced activation of ICAM-1 on endothelial cells from rats (Yasuma et al., 1997), the effect of temperature has not been taken into account and even seems to give contrasting results. Retention of lymphocytes in peripheral tissues might be a reasonable explanation for the rapid and fully reversible lymphopenia during torpor. However, more research is needed to obtain information about potential retention mechanisms during torpor *in vivo*.

4. Conclusion

In conclusion, leukocyte and thrombocyte counts in hibernating European Ground Squirrels decrease dramatically during torpor from its onset onwards, while restoration of euthermic cell counts is fully accomplished shortly after arousal to euthermia. The unaffected hematocrit suggests that the extreme decrease in circulating cells is not due to a fluid shift during torpor, while the unaffected white pulp size of the spleen demonstrates no massive apoptosis of lymphocytes. Our study is the first to present the effect of torpor-bout duration, the rate and rapidity of reversal of leukopenia during torpor. These data are crucial for dissection of its underlying mechanism. Unraveling the underlying signaling pathways will not only enhance our fundamental knowledge of the immune system, but may also identify new immune modulating pathways and may thus be of major relevance for application in the setting of enhanced immune activation or thrombocyte aggregation.

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