Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels

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Prendergast, Brian J., David A. Freeman, Irving Zucker, and Randy J. Nelson. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. Am J Physiol Regulatory Integrative Comp Physiol 282: R1054–R1062, 2002. First published January 17, 2002; 10.1152/ajpregu.00562.2001.—Golden-mantled ground squirrels (Spermophilus lateralis) undergo seasonal hibernation during which core body temperature (Tb) values are maintained 1–2°C above ambient temperature. Hibernation is not continuous. Squirrels arouse at ~7-day intervals, during which Tb increases to 37°C for ~16 h; thereafter, they return to hibernation and sustain low Tbs until the next arousal. Over the course of the hibernation season, arousals consume 60–80% of a squirrel’s winter energy budget, but their functional significance is unknown and disputed. Host-defense mechanisms appear to be downregulated during the hibernation season and preclude normal immune responses. These experiments assessed immune function during hibernation and subsequent periodic arousals. The acute-phase response to bacterial lipopolysaccharide (LPS) was arrested during hibernation and fully restored on arousal to normothermia. LPS injection (ip) resulted in a ~1–1.5°C fever in normothermic animals that was sustained for >8 h. LPS was without effect in hibernating squirrels, neither inducing fever nor provoking arousal, but a fever did develop several days later, when squirrels next aroused from hibernation; the duration of this arousal was increased sixfold above baseline values. Intracerebroventricular infusions of prostaglandin E2 provoked arousal from hibernation and induced fever, suggesting that neural signaling pathways that mediate febrile responses are functional during hibernation. Periodic arousals may activate a dormant immune system, which can then combat pathogens that may have been introduced immediately before or during hibernation.

**MAINTENANCE OF IMMUNE FUNCTION requires considerable energy expenditure (42, 62) and may limit or constrain the extent to which animals engage in other energetically demanding activities (39, 40, 58). Large seasonal increases in energy expenditure associated with reproduction or low ambient temperatures (5) may compete with the day-to-day demands of host defense. Sustained regulated hypothermia, in the form of hibernation or daily torpor, has been adopted by some mammals to contend with seasonal changes in temperature and energy availability. Golden-mantled ground squirrels (Spermophilus lateralis) spend 5–6 mo each year in deep hibernation, during which core body temperature (Tb) is maintained at 1–2°C above ambient temperature (Tamb). Many physiological systems, including brain and renal metabolism, respiration, cardiac function, digestion, and mitosis, are arrested or substantially reduced during a hibernation bout (12, 29, 31, 51, 54, 70, 74).

Although immune function during hibernation has been little studied, robust cell-mediated and humoral immune responses to pathogens are thought to be compromised. Impaired immune function in hibernators has been inferred from in vitro splenocyte proliferation or reduced antibody production over the course of an entire hibernation season (4, 7, 15, 32, 46, 48, 60). We are unaware of any studies that have assessed a hibernating individual’s acute response to infection during a hibernation bout.

During the hibernation season mammals are not continuously torpid, but instead arouse to normothermic Tbs (~37°C) at regular intervals, ranging from 2 to 30 days in different species. They sustain elevated Tbs for <1 day, after which they return to hibernation (21, 53, 71). Up to 80% of a hibernating mammal’s winter energy budget is consumed during these periodic arousals (9, 35, 66), and the consequent depletion of energy stores may contribute to overwinter mortality (71). The functional significance of periodic arousals has proven enigmatic: hypotheses that arousals are required to clear metabolic waste (16), replenish blood glucose (20), restore cellular electrolyte balance (17), regenerate the gonads (1), prevent muscular atrophy (68), or eliminate sleep debt (10, 56) have been proposed. Each of these hypotheses has been challenged or

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METHODS

Animals. Adult male and female golden-mantled ground squirrels (n = 31) born in the laboratory to field-caught pregnant dams, trapped in Donner State Park, Truckee, CA, at an altitude of 1,783 m, were housed from birth in a light-dark cycle that provided 14 h light/day (lights on: 0700 Pacific Standard Time) at a T_b of 21 ± 3°C. Squirrels were provided ad libitum access to water and food (Simonsen rat chow, maintenance diet). Animals were weighed (±0.1 g) before the initiation of all treatments.

Temperature telemetry. Body temperature was recorded telemetrically from temperature-sensitive radiotransmitters implanted intraperitoneally under deep surgical anesthesia (12.5 mg pentobarbital sodium per initial 100 g body mass ± 0.5 mg per each additional 10 g body mass) (11). After recovery from surgery, squirrels were individually housed at a T_b of 5 ± 3°C in cages placed on receiver boards: T_b data collected every 10 min were transmitted to and stored by a computer for subsequent analyses. The onset of a hibernation bout was defined as the time at which T_b decreased below 33°C and remained below this value for six consecutive hours. A hibernation bout was considered terminated, when Tb increased by >1°C during each of three consecutive 10-min intervals. The normothermic interval between hibernation bouts began when T_b exceeded 33°C and remained above this value for six consecutive hours. The duration of a periodic arousal was calculated as the interval between the onset of normothermia and the onset of the subsequent hibernation bout. Normothermic squirrels were classified as nonhibernators if they failed to exhibit a bout of hibernation after 2 wk at 5°C.

Intraperitoneal injections. All solutions, syringes, and needles were prechilled to 5 ± 3°C before the injections were delivered. Normothermic (T_b > 33°C; n = 10) and hibernating (T_b ≤ 33°C; n = 21) squirrels were injected intraperitoneally with E. coli LPS (Sigma Chemical, 0111:B4; 500 μg/kg in 0.1 ml sterile saline) or an equal volume of sterile saline between 1400 and 1800. Each normothermic squirrel received a control (saline) and an LPS injection on separate occasions in a blocked experimental design. Hibernating squirrels had been torpid for 38.1 ± 7.7 h (LPS) and 38.5 ± 12.2 h (saline) at the time of injection. Care was taken to minimize disturbance of hibernating squirrels during the injection procedure so as not to provoke premature arousal from hibernation. Bedding was cleared away by hand, exposing a hibernating squirrel’s lateral lower abdomen. A sterile 26-gauge hypodermic needle (connected to 9 cm of polyethylene infusion tubing and a 1.0-ml syringe) was inserted slowly through the peritoneum. Hibernating squirrels were observed for several minutes to ensure that insertion of the needle did not immediately provoke arousal from hibernation before injections were delivered. Normothermic squirrels were briefly restrained by hand and injected intraperitoneally with LPS or saline directly from a 1.0-ml hypodermic syringe.

Intracerebroventricular infusions. Normothermic squirrels (n = 19) implanted with radiotransmitters were fitted with stainless steel guide cannulas under deep surgical anesthesia (pentobarbital sodium). Cannulas (21 gauge) were stereotaxically implanted into the third ventricle (midline coordinates with ear bar at zero: 8.2 mm anterior to bregma and 7.3 mm below dura). After 7–10 days of postoperative recovery at 21°C, all squirrels were housed at 5°C. Infusion cannulas of hibernating squirrels were accessed by moving cage bedding to expose the animal’s head. A 26-gauge internal infusion cannula connected to a 10-μl Hamilton syringe was inserted into the guide cannula and chilled (5 ± 3°C) PGE_2 (Sigma Chemical; 500 ng in 10 μl; n = 7) or sterile saline (n = 10) was infused over the course of 60 s into the third ventricle. Squirrels had been torpid for 31.2 ± 6.3 h (PGE_2) and 31.7 ± 7.4 h (saline) at the time of infusion. All treatments and surgical procedures were approved by the University of California at Berkeley Animal Care and Use Committee.

Statistica. T_b data were collected for at least 1 wk before the initiation of treatments in each individual. The onset and duration of periodic arousals were calculated, as well as mean T_b at various intervals during hibernation, emergence, and arousal. In cases where T_b values were unavailable at a given time point, the arithmetic mean of the immediately preceding and succeeding T_b values was interpolated and used in statistical analyses. Data are presented as means ± SE. Standard parametric statistical analyses (between-subjects and repeated-measures ANOVA, unpaired and paired t-tests) were conducted using Statview 5.0 (SAS Institute, Cary, NC). Where significant F-ratios were obtained, pairwise comparisons were conducted using Fisher’s protected least significant difference test, or paired t-tests, as appropriate.

RESULTS

Effects of LPS in normothermic squirrels. LPS injections significantly increased core T_b of normothermic
ground squirrels over the first 12 h after treatment (ANOVA, $P < 0.0001$; Fig. 1). After handling-induced hyperthermia abated (hours 0 to +2), squirrels injected with LPS manifested a 1–1.5°C fever of >8-h duration, beginning 3–4 h after treatment; saline-treated squirrels, in contrast, exhibited no signs of fever but instead manifested the normal nocturnal decline in $T_b$ typical of this diurnal species (e.g., Ref. 18) and sustained $T_{bs}$ of 36–37°C during this interval (Fig. 1). $T_{bs}$ of LPS-treated squirrels were significantly higher than those of saline-treated squirrels for over 4.5 h of the initial 12-h posttreatment interval ($P < 0.05$ for each 10-min interval; Fig. 1). Unlike other stressors (e.g., repeated social defeat; Ref. 49), which result in long-term (days 12-h posttreatment interval ($P < 0.05$ for each 10-min interval), $T_{bs}$ of LPS-treated squirrels returned to values comparable to those of saline-treated squirrels within 24 h after treatment (data not shown).

**Effects of LPS in hibernating squirrels.** In contrast to normothermic squirrels, hibernating squirrels did not exhibit fever in the first few hours after treatment with LPS. The majority of squirrels (17 of 22) continued to hibernate after LPS and saline treatments and did not initiate a periodic arousal until >2 days after treatment. Saline- and LPS-treated squirrels did not differ in the latency to onset of periodic arousal ($P > 0.05$; Fig. 2A). On arousal from hibernation, LPS-treated squirrels exhibited significantly elevated $T_{bs}$, comparable to those observed in nonhibernating squirrels injected with LPS ($P < 0.05$ vs. saline-treated squirrels; Fig. 2B). Squirrels treated with saline had $T_{bs}$ of 37–38°C during the first 8 h of the subsequent periodic arousal (Fig. 2C), whereas squirrels injected with LPS sustained $T_{bs}$ that were ~1°C higher throughout most of this interval (ANOVA, $P < 0.01$; Fig. 2C). $T_{bs}$ of LPS-treated squirrels were significantly higher than those of the saline-treated squirrels (Fig. 2C) and significantly higher than their own $T_{bs}$ during previous arousals (i.e., before treatments; Fig. 2B). LPS treatments also significantly lengthened the duration of the subsequent periodic arousal; normothermia was sustained for 97 and 16.3 h, respectively, in squirrels treated with LPS and saline during hibernation ($P < 0.05$; Fig. 3).

**Effects of PGE$_2$ in normothermic and hibernating squirrels.** Central (icv) infusions of PGE$_2$, but not saline, elicited $T_b$ increases of 1–2°C. This acute, monophasic hyperthermia was sustained for <2 h after PGE$_2$ treatment (Fig. 4; $P < 0.05$ vs. saline-treated squirrels for each 10-min interval). In hibernating squirrels, PGE$_2$ treatments did not elicit comparably rapid hyperthermia (not illustrated) but tended to accelerate emergence from hibernation (Fig. 5A). Hibernating squirrels treated with PGE$_2$ did not emerge from hibernation immediately; instead, they initiated periodic arousals ~16 h after treatment, compared with a 49-h latency for squirrels treated with saline ($P = 0.06$; Fig. 5A). PGE$_2$ infusions delivered during hibernation first induced fever after the squirrels next aroused (Fig. 5, B and C). $T_{bs}$ of PGE$_2$-treated squirrels during the immediate posttreatment periodic arousal were significantly higher than those of saline-treated squirrels and significantly higher than their own $T_{bs}$ during a pretreatment periodic arousal ($P < 0.05$, both comparisons; Fig. 5B). As in nonhibernating animals, PGE$_2$ treatment of hibernators was associated with a 1.5–2.5°C increase in $T_b$ that was sustained for ~3 h after arousal (for each 10-min interval, $P < 0.05$ vs. saline value and $P < 0.05$ vs. pretreatment value; Fig. 5C). Squirrels also remained normothermic on average 3.5 times longer after PGE$_2$ treatment than after saline treatment; however, this difference was not statistically significant ($P = 0.07$; Fig. 5D).

**DISCUSSION**

These experiments assessed immune function in hibernating and normothermic ground squirrels. In nonhibernating squirrels, LPS, a nonreplicating pathogen that simulates infection, elicited a 1–1.5°C increase in $T_b$ within 4 h; this fever persisted for >8 h. Hibernating squirrels, in contrast, initially exhibited no discernible febrile response to LPS. $T_b$ of hibernating squirrels treated with LPS remained low (5–10°C) during the immediate posttreatment interval, and normal hibernation bouts were sustained and terminated spontaneously ~2 days after LPS treatment. Within 1 h of arousal from hibernation, however, LPS-treated squirrels exhibited a fever of 1–1.5°C, which was sustained for >8 h. The inability of LPS to induce fever while
squirrels are in hibernation may indicate that component(s) of the APR to systemic infection (14, 38) are disabled at low $T_b$s but are reactivated on return to normothermic $T_b$s.

One or more of the cellular and humoral signaling events involved in the APR may be interdicted in hibernating squirrels. In normothermic mammals, LPS treatment induces fever by triggering secretion of cytokines and other humoral mediators from macrophages and monocytes at the site of infection (3, 38). The cytokines IL-1β, IL-6, and tumor necrosis factor-α mimic, to varying degrees, LPS-induced fever in mice, rats, and rabbits; inhibition of these cytokines attenuates LPS-induced fevers (38). LPS and cytokines do not cross the blood-brain barrier, but they may act within blood vessels, at the meninges, or at circumventricular sites to produce diffusible mediators (63), which in turn trigger hyperthermia by their action on temperature-sensitive neurons in the preoptic area (POA; Ref. 34). Injections of cyclooxygenase inhibitors to the POA prevent LPS-induced fevers, indicating that PG activity at this site may be the final endocrine mediator of LPS-induced fever (57).

Low $T_b$s characteristic of hibernation may preclude fever by inhibiting endocrine and immune function at any of a number of stages of the febrile cascade, e.g., encounters between target cells and LPS, LPS binding or internalization, LPS induction of cytokine production, or cytokine induction of PG secretion. Torpor is accompanied by reduced blood pressure and circulation (30), and the proportion of leukocytes is substantially reduced during hibernation (72). Temperature effects on membrane LPS binding are complex and do not lend themselves to simple interpretation: depending on the

Fig. 2. Nonresponsiveness to LPS during hibernation and LPS-induced fever during subsequent periodic arousal. Hibernating golden-mantled ground squirrels were injected intraperitoneally with 500 μg/kg LPS or sterile saline. A: time interval between injection and the initiation of a periodic arousal. Values are means ± SE. B: $T_b$ during the first 2 h of the periodic arousal exhibited before and after treatment with LPS or saline. Values are means ± SE. *$P < 0.05$ vs. saline-treated squirrels. C: $T_b$ during each 10-min interval over the first 8 h of the periodic arousal immediately preceding (Previous) and after treatment with LPS or saline. Values are means ± SE. Solid bars along the abscissa indicate 10-min intervals during which $T_b$ of LPS-treated squirrels significantly exceeded that of either saline-treated squirrels or squirrels during the preceding (pretreatment) periodic arousal ($P < 0.05$).
strain of LPS and the host cell type and source, low temperatures (4–10°C) can inhibit (22, 24, 37, 67) or have no effect (28, 37, 67) on in vitro LPS binding and motility. However, low temperatures reliably inhibit internalization of LPS (36, 67), as well as potentiators of LPS-induced cytokine release (e.g., heparin-binding protein; Ref. 28). Temperature dependence of LPS internalization may account for observations that low temperatures inhibit LPS-induced cellular responses (phagocytosis, Ref. 41; endothelial cell detachment, Ref. 26; clearance of immune complexes, Ref. 55). Cytokine synthesis may also be inhibited by low temperatures (73). Finally, the ability of proinflammatory cytokines to stimulate target cells may also be temperature-sensitive: IL-1α and IL-1β are not internalized by articular chondrocytes at 4°C and are more potent stimulators of PGE2 synthesis at 37°C than at 4°C (8). A decrease in temperature from 36°C to 34°C completely eliminates the ability of IL-1 to stimulate thymocyte mitosis (25). Low tissue temperatures generally inhibit the processing of fever signals at any of several stages in the febrile cascade and may account for the observed nonresponsiveness to LPS of ground squirrels in deep torpor. Although the latency between PGE2 treatment and Tb elevation was greater in torpid relative to normothermic squirrels, hibernating squirrels were nonetheless responsive to central infusions of PGE2, as evidenced by the acceleration of arousal from torpor after PGE2 infusions. This suggests that eicosanoid receptors and postreceptor signaling mechanisms are functional at low Tbs. Thus the physiological basis of nonresponsiveness to LPS during a hibernation bout appears to be located upstream from PG receptors in the LPS-fever pathway, perhaps at the level(s) of LPS induction of cytokine synthesis or cytokine induction of PG secretion.

The present findings extend earlier work suggesting that organismal immune responses are inhibited, to varying degrees, during hibernation. For example, antibody production in response to sheep red blood cells and hen egg white lysozyme was diminished in hibernating 13-lined ground squirrels (32) and Syrian hamsters (6), respectively, as were in vitro agglutination responses of splenocytes derived from hibernating Syrian hamsters (60). Hibernation delays rejection of skin allografts (59), growth of neoplastic homologous tumors (45), and progression of coxsackie B3 viral infection (13), and it retards the development of cellular damage after lethal doses of x-irradiation (4). Each of these challenges, however, required intervals of days to weeks to elicit measurable immune responses, an interval that encompasses several periodic arousals and hibernation bouts. Such challenges, therefore, do not permit inferences regarding immunocompetence during a given hibernation bout.

In contrast, the present experiment used a pathogen that elicits a primary immune response over a time frame of a few hours; moreover, this challenge was
Fig. 5. PGE2-induced acceleration of re-warming, induction of fever, and lengthening of periodic arousal duration in hibernating ground squirrels. 

A: Time interval between PGE2 (500 ng in 10 μl, 60 s) or saline (10 μl) infusion and arousal from hibernation. (P = 0.06). Values are means ± SE. 

B: Tb during the first 2 h of the periodic arousal immediately preceding and after treatment with PGE2 or saline. Values are means ± SE. 

C: Tb during the first 8 h of the periodic arousal exhibited immediately preceding and after treatment with PGE2 or saline. Values are means ± SE. Solid bars along the abscissa indicate 10-min intervals during which Tb of PGE2-treated squirrels significantly exceeded that of either saline-treated squirrels or their own Tb during the preceding (pretreatment) periodic arousal (P < 0.05). 

D: Duration of the periodic arousal immediately after PGE2 and saline treatments (P = 0.07). Values are means ± SE.
presented during a hibernation bout. No integrated febrile response occurred during the hibernation bout; immediately on arousal from hibernation, however, LPS-treated squirrels exhibited fever, indicative of responsiveness to the LPS treatments administered several days earlier. The magnitude of these fevers was comparable to that elicited in nonhibernating squirrels, suggesting that the febrile response to pathogens is equivalent in nonhibernating squirrels and hibernators undergoing a periodic arousal. It seems unlikely, therefore, that the changes that render hibernating squirrels unresponsive to LPS are a consequence of a seasonal program or rhythm of responsiveness to pathogens, as previously suggested (e.g., Ref. 61); rather, the highly labile and reversible hyporesponsiveness to LPS likely reflects masking by low TbS in vivo. Thus arousal from hibernation appears necessary for the expression of febrile responses to LPS.

Despite their substantial energetic cost (9, 35, 66), periodic arousals are ubiquitous among mammalian hibernators, and numerous hypotheses have been advanced to explain their functional significance (71). In most instances, arousals are viewed as emerging from constraints imposed on animals that sustain low TbS for extended intervals; the periodic normothermia is viewed as necessary for removal of this, as yet to be identified, constraint. The abnormally long intervals of normothermia during the initial arousal after LPS treatment may be related to incompatibility of immune activation and anaptyrexia, and they are consistent with anecdotal accounts that “sick animals never enter hibernation” (Ref. 44, p. 196–197). This observation, coupled with the finding that responsiveness to bacterial pathogens is arrested during hibernation, but fully restored on arousal, suggests that periodic arousals permit reactivation of a dormant immune system that can monitor for bacterial infection acquired during the preceding hibernation bout. Few studies have addressed immune function specifically during periodic arousals, but available data indicate that multiple, brief returns to normothermia exacerbate the effects of viral infections in hibernators. Sublethal doses of coxsackie B3 virus killed hibernating ground squirrels, with the progression of the infection restricted to periodic normothermic intervals (13). Thus the immune system appears incapable of successfully contending with viruses during the hibernation season. In contrast, activation of the APR in aroused hibernators and induction of fever during a periodic arousal suggest that animals are capable of responding to a bacterial infection in a normal manner during normothermic intervals. This contrast illustrates the potential immunological trade-offs associated with the execution of periodic arousals: such arousals may be required to monitor for and respond to bacterial pathogens while at the same time exacting a cost in potentiating the progression of viral infections.

Our data suggest that bacterial pathogens introduced during a hibernation bout do not provoke arousal from deep torpor or immune responses. Low TbS do not, however, deter bacterial growth and prolif-
eration. Indeed, injection of hibernating 13-lined ground squirrels with large numbers of Mycobacterium leprae results in rapid generalized infection and death, despite low TbS (19). After brief (4–8 h) periods of adaptation, many species of bacteria, including E. coli (65), Campylobacter jejuni (27), Salmonella heidelberg (64), S. enteritidis (33), Listeria monocytogenes (2), Bacillus subtilis (69), and Vibrio vulnificus (47), continue to exhibit exponential growth at 5–10°C, temperatures well within the range of mammalian hibernation. Periodic elevations in TbS appear to be necessary for recognition of introduced pathogens, initiation of the APR response, maintenance of fever, and phagocytosis to avoid colonization and the ground squirrel’s eventual death. The marked energetic costs of periodic arousals may in part reflect a trade-off between long-term requirements of maintaining energy balance and the need to contend with the more proximate challenge of parasite colonization.

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