Cell

Circannual Control of Hibernation by HP Complex in the Brain

Noriaki Kondo,[1,2,*] Tsuneo Sekijima,[2,6] Jun Kondo,[2,3] Nobuhiko Takamatsu,[2,4] Kazuo Tohya,[5] and Takashi Ohtsu[2,7]
[1] Mitsubishi Kagaku Institute of Life Sciences
[2] Kanagawa Academy of Science and Technology (KAST)
Machida, Tokyo 194-8511, Japan
[3] Mitsubishi-Tokyo Pharmaceuticals Inc. Yokohama Research Center, Kanagawa 227-0033, Japan
[4] Department of Biosciences, School of Science, Kitasato University, Kanagawa 228-8555, Japan
[5] Department of Anatomy, Kansai College of Oriental Medicine, Osaka 590-0482, Japan
[6] Present address: Graduate School of Science and Technology, Niigata University, 2-8050 Ikarashi, Niigata 950-2181, Japan.
[7] Present address: Kanagawa Cancer Center Research Institute, 1-1-2 Nakao, Asahi-ku, Yokohama 241-0815, Japan.
*Contact: nkondo@libra.ls.m-kagaku.co.jp
DOI 10.1016/j.cell.2006.03.017

SUMMARY

Seasonal hibernation in mammals is under a unique adaptation system that protects organisms from various harmful events, such as lowering of body temperature (Tb), during hibernation. However, the precise factors controlling hibernation remain unknown. We have previously demonstrated a decrease in hibernation-specific protein (HP) complex in the blood of chipmunks during hibernation. Here, HP is identified as a candidate hormone for hibernation. In chipmunks kept in constant cold and darkness, HP is regulated by an individual free-running circannual rhythm that correlates with hibernation. The level of HP complex in the brain increases coincident with the onset of hibernation. Such HP regulation proceeds independently of Tb changes in constant warmth, and Tb decreases only when brain HP is increased in the cold. Blocking brain HP activity using an antibody decreases the duration of hibernation. We suggest that HP, a target of endogenously generated circannual rhythm, carries hormonal signals essential for hibernation to the brain.

INTRODUCTION

One of the most curious biological phenomena in mammals is their ability to hibernate circannually, which allows them to survive unusually low body temperatures (Tb) at or near 0°C. As lowering Tb brings about dysfunction of various cells and organs, such as heart and brain, hibernators are required to develop a capacity for maintaining regulated functions at potentially lethal low Tb during hibernation. It has already been shown that during hibernation, organisms are protected not only from hypothermia (Hochachka, 1986) but also from ischemia (Frerichs and Hallenbeck, 1998), muscle disuse (Harlow et al., 2001), bacterial infection (Sharapov, 1983), and tumorigenesis (Kemper and Ruben, 1982), suggesting that hibernation is a unique physiological adaptation for preventing lethal damage and diseases caused by various harmful events. Such an adaptation can be assumed to be accomplished prior to the onset of and sustained during hibernation because central and peripheral organs would rapidly lose functions at low Tb if the adaptation were not accomplished in advance. In fact, in the heart of chipmunks, a rodent hibernator, the capacity of intracellular stores to take up cytosolic calcium ions, which is critical for avoiding intracellular calcium overload evoked at low Tb (Hochachka, 1986), is greatly enhanced prior to the onset of and during hibernation (Kondo and Shibata, 1984; Kondo, 1986a, 1987, 1988). This enhancement is also caused throughout the hibernation season even in animals prevented from experiencing a lowered Tb by keeping them constantly warm (Kondo, 1987; Kondo and Kondo, 1992b), indicating that cellular adjustment in the heart occurs on a seasonal basis without a lowering of Tb. Furthermore, several physiological and molecular changes, such as decreased locomotor activity and food intake, depressed endocrine functions (Wang, 1982), and the regulated expression of a few proteins (Kondo and Kondo, 1992a; Srere et al., 1992), have been seasonally observed before the beginning of hibernation. These studies suggest the existence of a systemic adaptation mechanism for hibernation independent of Tb changes, which led us to consider the possibility of a seasonally controlled molecular network through which the functions of principal organs would be modulated for maintaining the organism in a healthy state during the hibernation season. For decades, many studies have been carried out to explore factors responsible for hibernation that are seasonally regulated, especially hormones which play a central role in

physiological adaptation to various internal and external changes (Wang, 1982, 1988). However, no factor absolutely critical for hibernation has been discovered.

In a previous study, we found a protein complex (140 kDa MW) specific for hibernation (hibernation-specific protein, HP) in the blood of chipmunks, as a complex that was decreased in the blood during hibernation (Kondo and Kondo, 1992a). This complex is composed of four proteins, three of which are structurally homologous proteins with a collagen-like domain in the N-terminal regions (HP20, 25, and 27) and form a complex called HP20c by triple-helix formation of this domain. In blood, HP20c is further associated with the fourth protein, HP55, which is homologous to α 1-antitrypsin, a member of the serpin superfamily. HP20c associated with HP55 (HP complex, HPc) is known to be produced specifically in the liver and secreted into the blood (Takamatsu et al., 1993, 1997).

Herein, we demonstrate that HP complex, whose concentration in the blood and brain is regulated by an endogenously generated circannual rhythm, is a candidate hormone that may be essential for hibernation.

RESULTS

Free-Running Hibernation Rhythm in Chipmunks

Although an exact characterization of hibernation rhythm is of prime importance for studying the mechanisms of circannual control of hibernation, there is little evidence for its rhythmicity under constant laboratory conditions throughout life. Therefore, in 27 male chipmunks kept under constant cold (5°C) and dark conditions throughout their lives, the precise rhythmicity was examined by monitoring surface Tb changes using a method we originally developed for determining the accurate onset, termination, and duration of hibernation (Figure 1). The characterization and evaluation of hibernation were based on this method throughout the study. Twenty of twenty-seven animals exhibited a clear hibernation rhythm with individually constant cycles and durations (Figure 2A), whereas the other seven animals (26% of animals tested) never hibernated until they died naturally within a few years. The individual hibernation rhythm was sustained throughout their life spans, the maximum of which was 11 years, a duration surprisingly longer than that of rats (about 4-fold longer). The mean period of the rhythm was 313 days, and the maximum and minimum periods were 391 and 157 days, respectively (Figure 2B). The period varied widely between animals but was less than a year in most (17/20 animals). Such a period shorter than a year has been observed in golden-mantled ground squirrels kept in the cold with a 12 hr LD photoperiod (Pengelley and Asmundson, 1974). Thus, in chipmunks, hibernation is strictly controlled by an individual circannual rhythm throughout life under constant cold conditions. Interestingly, the timing and duration of hibernation is little affected by aging under laboratory conditions.

Circannual Regulation of HP Complex (HPc) Correlates with Hibernation

As the results of a previous study showed that HPc levels in the blood were markedly decreased during hibernation (Kondo and Kondo, 1992a), the relationship between HPc changes in the blood and circannual hibernation rhythms was examined throughout the entire life of the animal. HPc levels in blood collected monthly from the above 27 animals kept in the cold and dark were analyzed. In 20 animals exhibiting hibernation rhythm, the HPc concentration in the blood (479 ± 38 nM; n = 13) started to decrease prior to the onset of and remained low during hibernation (76 ± 19 nM; n = 13), following which hibernation was terminated with an increase in HPc (Figure 2C). Such an association between HPc and hibernation was sustained throughout their lives. However, the seven animals that never underwent hibernation did not exhibit an HP rhythm even though HPc was present at normal levels in the blood (512 ± 56 nM; n = 7; Figures 2D and 2E). Thus, the regulation of HPc in the blood appears to correlate with circannually controlled hibernation throughout life.

Circannual Regulation of HPc Is Independent of Tb Changes

In order to clarify whether a decrease in HPc that correlates with hibernation is due to low Tb during hibernation or not, HP rhythm in the blood was examined in animals in which a decreased Tb was prevented by keeping them under conditions of constant warmth (23°C) and a 12 hr LD cycle. Among 29 animals in which a shallow torpor was not detected (see Experimental Procedures), 22 generated circannual HP rhythms (Figure 3A) while the remaining seven had no HP rhythm, like the animals unable to hibernate (see Figure 2D). The mean period of HP rhythm between peaks detected by Western blotting of blood collected monthly, which corresponds to that of hibernation rhythm, was 10 months (Figure 3B). The maximum and minimum periods were 13 and 6 months, respectively. These rhythm characteristics were similar to those in animals with a low Tb during hibernation season, suggesting that HPc is regulated by endogenous circannual rhythms responsible for hibernation and not by changes in Tb and/or environment and that the timing of this rhythm may be insensitive to Tb changes.

To examine whether the physiological state during HPc reduction allows organisms to lower Tb as hibernating animals do, animals with or without decreased HPc levels were exposed to cold conditions (5°C). Whenever a circannual decrease in HPc in the blood was attained, Tb was lowered within a few days (1.43 ± 0.25 days; n = 10) after the cold exposure (Figure 3C). However, the cold conditions never lowered Tb without decreasing HPc. In fact, a few animals that did not generate HP rhythm throughout their life underwent neither low Tb nor a decrease in HPc even by prolonged exposure to cold and died within a few years (data not shown). These results indicate that only during regulated reduction of HPc did the physiological state allow the organisms to survive low Tb. The timing

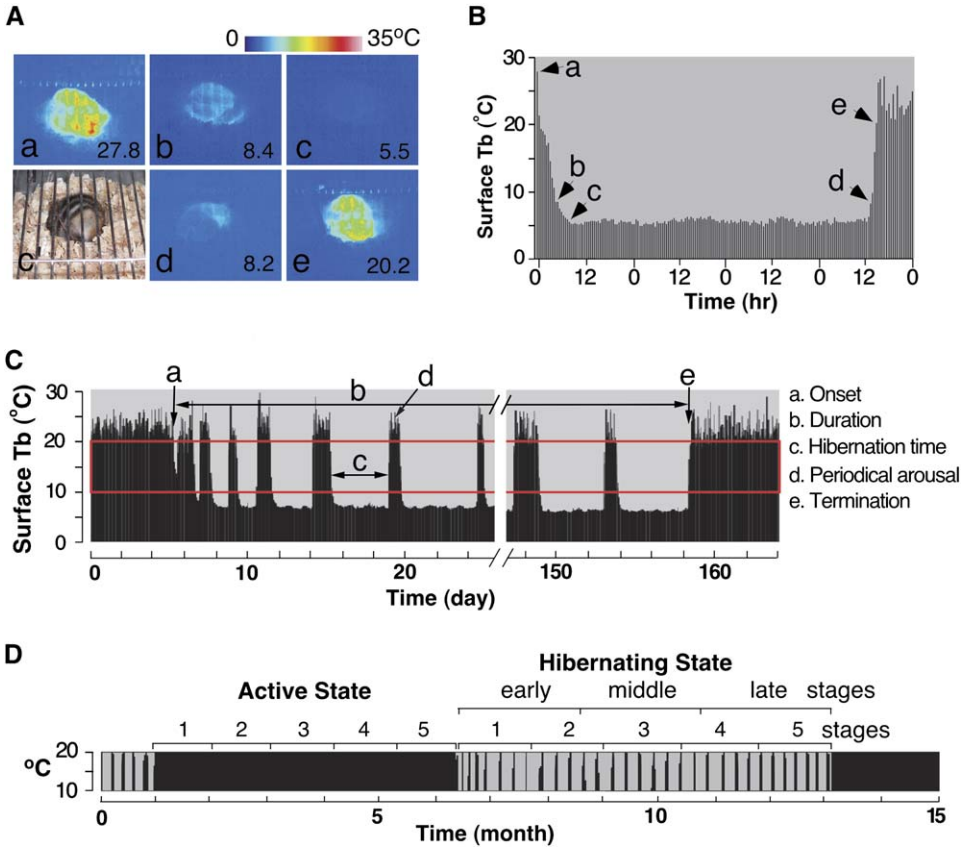

Figure 1. Interpretations of Tb-ms Recordings by Computer-Controlled Thermal Video System and Definition of Stages in Hibernation Rhythm

(A) Actual images taken for Tb-ms measure. Thermal images (a–e) and picture (c′) corresponding to (c) were taken at arrows in (B). Number in images: Tb-ms used for recordings.

(B) Tb-ms recording at 30 min intervals (each bar) for 4 days between periodical arousals during hibernation. Arrows: thermal images in (A).

(C) The onset, duration, and termination of hibernation, and periodical arousal were detected in Tb-ms recording (a, b, e, and d). Hibernation time (c) was defined as duration of time when Tb-ms was below 10°C. Tb-ms recording between 10°C and 20°C (red frame) was used in (D) as well as Figures 4A and 6A.

(D) Each active and hibernating state was divided into five stages for statistical analysis in Figure 4B. The hibernating state was redefined as the early, middle, and late stages for examining the effects of the antibody in Figure 6.

and period of physiological adaptation to hibernation correlate with HPc regulation independently of Tb changes, suggesting a molecular involvement of circannually regulated HPc in adaptation to hibernation.

Circannual Rhythm of HPc Is Generated by Gene Expression

The gene expression of HP was examined in relation to HPc changes in the blood to clarify how HPc is decreased in the blood even in animals maintaining a high Tb (Figure 3D). As expression of HP20, 25, and 27 is known to be repressed in the liver during hibernation (Takamatsu et al., 1993), their mRNAs in the liver were analyzed in animals kept under cold or warm conditions. In the cold, the amount of the *HP* mRNAs were decreased in association with HPc reduction in the blood despite no definitive changes in *albumin* mRNA, and these decreases were triggered prior to the onset of hibernation. A similar association between the mRNAs and HPc level in the blood was revealed in animals without a low Tb housed in constant warmth. These results indicate that HPc reduction in the blood is due to the downregulation of liver HP expression controlled by endogenous circannual rhythms and not metabolic inhibition at low Tb.

Increase in HP Levels in the Brain

Although decreasing HPc production during the hibernation season implies the involvement of HP in the control of hibernation is negative, its importance in hibernation was considered because of the strict correlation of circannual HP regulation to hibernation as shown in the above experiments and species-specific expression of *HP* genes

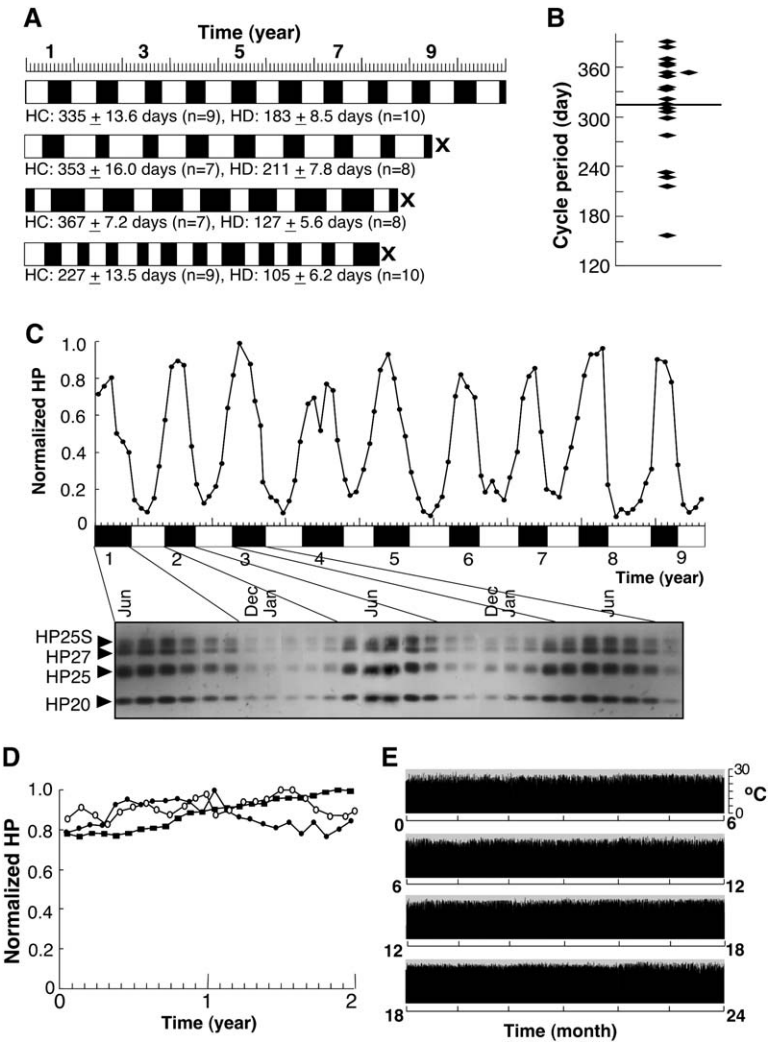

Figure 2. Animals with or without Free-Running Circannual Rhythm of Hibernation and Blood HP in Constant Cold (5°C, Darkness)

(A) Individual rhythms of active and hibernating states (closed and open columns) for 10 years in four animals determined by measuring Tb-ms as shown in Figure 1C. Numbers (mean ± SEM) under each column: hibernation cycle (period from the onset to the next onset of hibernation: HC) and duration (HD). X: animals died.

(B) Cycle periods of hibernation (days) in 20 animals. These values were obtained as shown in (A). Bar: mean of these values.

(C) Rhythm in HP (top graph) and hibernation (middle column). HP content in plasma collected monthly was determined by Western blotting (bottom panel) and normalized to its maximum content. The normalized values were plotted as a function of time (month) in the top graph. Arrowheads in Western blots: positions of HP25S, 27, 25, and 20. HP25S: glycosylated HP25.

(D and E) HP changes in blood over 2 years in three of seven animals unable to hibernate and the representative Tb-ms recording as a function of recording time (E).

in hibernators in the squirrel family (Kondo and Kondo, 1992a, 1993; Takamatsu et al., 1993; Kojima et al., 2001). If necessary for hibernation, HP might be increased in regions where HP functions. We therefore hypothesized that HP might act in the brain. Cerebrospinal fluid (CSF) collected from lateral ventricles was analyzed in active and hibernating animals kept in the cold. In CSF, all components of HPc (HP20, 25, 27, and 55) were detected. Coincident with a circannual decrease in HPc in the blood, HP levels in CSF determined by measuring HP20, 25, and 27 in CSF were dramatically increased (Figures 4A and 4B). The amount of HP in CSF was significantly increased in CSF before the onset of hibernation and reached a peak at the middle stage of hibernation when HPc in the blood was the lowest. Even at this stage, HP levels in the blood were much higher than those in CSF (about 3% of blood HP). At the late stage, although HPc in the blood increases, HP in CSF abruptly decreases, coinciding with termination of hibernation. Animals unable to generate HP rhythm and express hibernation in the cold, as shown in Figure 2D, did not upregulate HP in CSF (see Figure S1 in the Supplemental Data available with this article online).

The upregulation of HP in CSF may not be due to expression of *HP* genes, since a predominant signal was not detected by real-time RT-PCR analysis in various regions covering total brain from hibernating animals (Figure 4C). In immunohistochemical analysis using affinity-purified anti-HP20c antibody, HP was detected in the cytoplasm of choroid plexus epithelium, and the signal intensified as hibernation progressed (Figure 4D). These observations suggest an increase in the amount of HP in the brain by active transport via the choroid plexus, known as the blood-CSF barrier and the major producer of CSF, although the possibility of HP expression below the detection limit in the brain cannot be ruled out.

Since a similar circannual regulation of HP in CSF occurred in animals without a low Tb in constant warmth (Figure 4E), it is evident that the proposed transport of HP into CSF through the blood-CSF barrier is facilitated

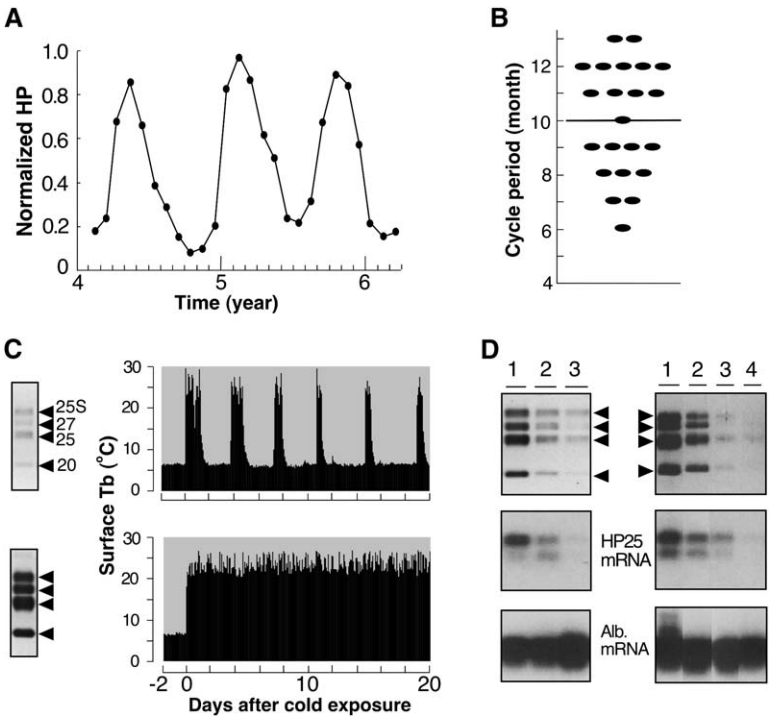

Figure 3. Circannual Regulation of Blood HP and Liver *HP* Gene Expression in Constant Warmth (23°C, 12 hr LD Cycle) and Induction of Hibernation by Cold

(A) Circannual HP changes in the blood over a 2 year period after 3 years in captivity. HP content was normalized as shown in Figure 2C.

(B) Cycle periods between peaks of free-running circannual HP rhythm in the blood (n = 22). Bar: mean of these values.

(C) Induction of hibernation by cold exposure in low and high HP states (upper and lower panels). Left and right panels: Western blots of plasma (arrowheads as shown in Figure 2C) collected just before the cold exposure and Tb-ms recordings for 20 days after cold exposure. Animals were placed in a cage on day 0.

(D) Circannual HP changes in the blood, liver *HP25*, and *albumin* mRNA (top, middle, and bottom panels). Left panels: active (1), prehibernating (2), and hibernating (3) states in constant cold. Right panels: animals in constant warmth sacrificed in June (1), December (2), and the following January (3) and February (4). For the other components of HP20c (HP20 and 27), a similar association was obtained (data not shown).

under control of a circannual rhythm independently of Tb changes.

HP Complex Is Dissociated in Brain

An association of HP20c (a complex of HP20, HP25, and HP27) with HP55 in the blood has previously been shown (Kondo and Kondo, 1992a). The HP complex in blood was compared to that in CSF using size exclusion chromatography. Although plasma HP20c coeluted with HP55 over several fractions, fractions of CSF containing HP20c did not overlap extensively with fractions containing HP55. Such a dissociation of the HP complex in CSF was supported by the results of immunoprecipitation using anti-HP27 antibody (Figure 5B). HP55 coimmunoprecipitated with HP20c from plasma, but was not present in immunoprecipitates of CSF. There were no changes in the molecular weights of HP20c components or HP55 between plasma and CSF (Figures 4B, inset, and 5). These findings suggest that some amount of the HP complex in the blood is transported into CSF in conjunction with dissociation of HP55 from the complex.

Anti-HP20c Antibody Decreases Hibernation Time

The functional importance of HP20c in hibernation was examined in the brain. The circannual timing and duration of hibernation measured in the preceding hibernation season were found to be relatively constant in individuals (Figure 2A). In order to block HP20c activity in CSF, polyclonal anti-HP20c IgG, which immunoprecipitates HP20c in the blood and CSF and interferes with the interaction between HP20c and HP55 in the blood (Figure 6B, inset), or control IgG, was administered into the lateral ventricles of hibernating animals for 2 weeks (Figure 6A). For quantitative analysis of hibernation, the amount of time spent in low Tb (hibernation time) during the 2 weeks of antibody administration was compared to that during the 2 weeks prior to antibody treatment. The administration of preimmune IgG during the early, middle, and late stages of hibernation had only a slight effect on hibernation time, while that of anti-HP20c IgG markedly decreased hibernation time. When the anti-HP20c antibody was administered in the early and middle stages, the decreased hibernation time recovered to normal levels after finishing administration in all animals tested (n = 5 in each), while antibody administration in the late stage accelerated the termination of hibernation in 2 of 5 animals (Figures 6Aa–6Ac). The effect of the antibody, a decrease in hibernation time, was dramatic in early and late stages and somewhat less at the middle stage (Figure 6B). This may be because the largest amount of HP20c in CSF was seen in this middle stage (Figures 4A and 4B).

The dose-inhibition relationship of anti-HP20c IgG on hibernation was examined in the latter half of the hibernation period (stages 3 to 4 in Figure 4B) where HP20c levels in CSF were relatively constant. Intraventricular administration of the IgG decreased the hibernation time in a dose-dependent manner (Figure 6C). The highest dose of IgG tested (0.045 mg/100 g body weight/day), where the hibernation time was shortened below 10% of control, was used in the experiments shown in Figures 6A and 6B. The antibody was reactive specifically to components of

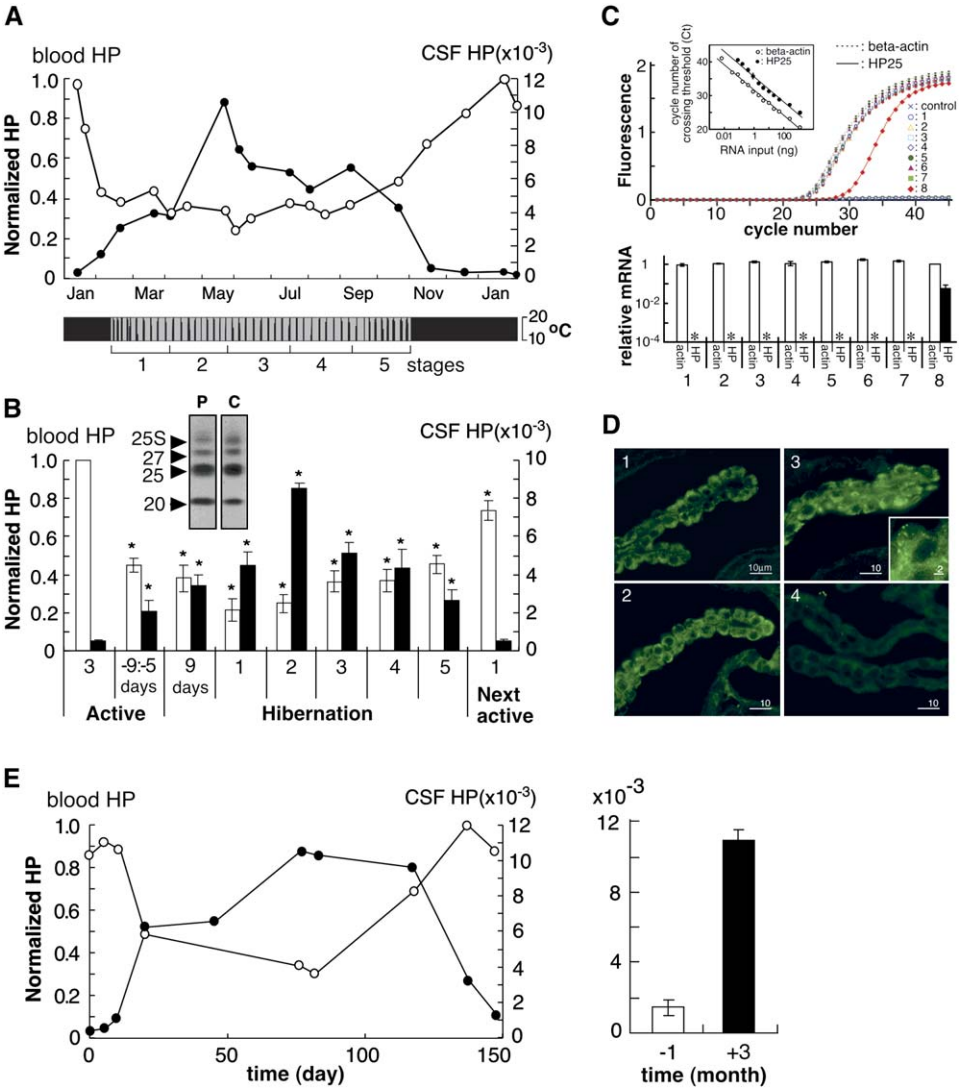

Figure 4. Circannual HP Transport into CSF Associated with HP Downregulation in Blood

(A and B) Representative result and statistical analysis (B) of HP changes in the blood (open symbols, left axes) and CSF (closed symbols, right axes) under conditions of constant cold and dark. Upper graph: HP changes in the blood and CSF over 13 months and the corresponding Tb-ms recording (attached column) as illustrated in Figure 1D. HP levels were normalized as shown in Figure 2C. In (B), active and hibernating states in six animals tested were divided into five stages as shown in Figure 1D and the attached column in (A). HP levels in the blood and CSF at each stage and 5–9 days before (−9:−5 days) and 9 days after (9 days) the onset of hibernation were normalized to the maximum HP content in the blood (stage 3 of active state) (mean ± SEM; n = 6). The asterisks of open and closed bars indicate significant differences compared with the corresponding values of stage 3 in active state. Inset panels: Western blots of HP20c components in plasma (P) and CSF (C) from the same animal.

(C) Real-time RT-PCR analysis of *HP25* mRNA expression in regions covering total brain (1, choroid plexus; 2, pituitary gland; 3, brain stem; 4, cerebellum; 5, diencephalon; 6, cerebrum; 7, olfactory bulb) and liver (8) of hibernating animals using *HP25* and β-*actin* gene-specific primers. Upper graph: normalized fluorescence (delta Rn) for these genes as a function of cycle number for PCR reactions and standard curves with 10^{-4} measurable range (inset; mean ± SEM; n = 3). Lower graph: relative ratio of mRNA levels of brain regions and liver to liver β-*actin* mRNA levels (mean ± SEM; n = 6). **HP25* mRNA levels in brain regions below the detection limit of this system.

(D) Localization of HP in the choroid plexus of chipmunks in the course of circannual cycle (1, active state; 2, onset; 3, middle stage of hibernation; 4, negative control without first antibody). Inset: remarkable HP-immunopositive granules in the basolateral to apical site of the choroidal cell cytoplasm in hibernating animal.

(E) HP changes in the blood and CSF (open and closed circles) normalized to the maximum HP content in the blood in constant warmth throughout (left graph), and HP in CSF one month before (−1) and 3 months after (+3) the beginning of HP upregulation (mean ± SEM; n = 5; right graph).

HP20c in CSF (Figure 6C, inset). The results show that blocking HP20c activity in the brain with antibody greatly decreases the hibernation time during which organisms are capable of lowering Tb and thus suggest a critical role for HP20c in the brain in developing a capacity for hibernation.

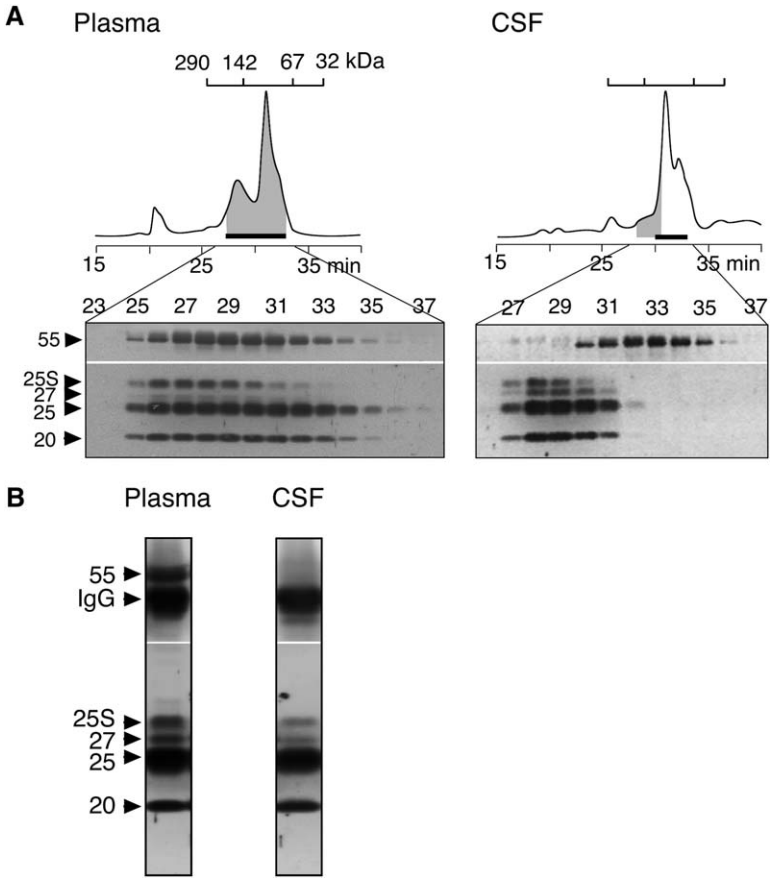

Figure 5. Difference between HP Complex in Blood and CSF

(A) Plasma and CSF (left and right panel) were separated by size exclusion chromatography and fractions collected every 30 s (upper panels, 15–40 min fractions). A portion of fractions 23–37 of plasma and 26–37 of CSF were analyzed by Western blotting (lower panels). Gray area and bar in each profile indicate elution areas of HP20c and HP55 (top arrowhead in the lower panel), respectively.

(B) Plasma and CSF (left and right panels) were immunoprecipitated with anti-HP27 antibody, and then a Western blot was performed with antisera against HP20, 25, 27, and 55. Arrowheads: each component of HP complex and anti-HP27 IgG.

DISCUSSION

Although attempts have been made for over half a century to identify substances responsible for hibernation in the blood and organs of hibernating animals (Amorese et al., 1982; Wang, 1988), there has been little success. In this study, the HP20 complex, HP20c, is identified as a promising candidate hormone controlling hibernation. The physiological and biochemical characteristics of the HP complex fit the major criteria for a hormone, for example, HP is produced in the liver and secreted in the blood (Kondo and Kondo, 1992a; Takamatsu et al., 1993), and here we provide evidence that expression of HP and transport into the brain are regulated by endogenous circannual rhythms. A critical role for HP20c in hibernation is supported here by the correlation between the increase in brain-HP20c and the timing and duration of hibernation and by the negative effect on the duration of hibernation resulting from administration of anti-HP20c antibody into the brain. Such a role for HP20c is further supported by evidence that Tb is lowered by cold exposure only when HP20c is increased in the brain and that animals lacking an increase in HP20c in the brain never express hibernation in the cold throughout their life.

Low Tb, during which physiological and biochemical functions are markedly depressed, has been used as the only marker of hibernation, which has been defined as a state where an animal has a Tb of about 5 degrees centigrade for more than a day. This definition has made it very difficult to distinguish physiological, biochemical, and molecular changes due to low Tb from changes due to endogenous rhythms independent of Tb. Although HP production in the liver has been shown to be depressed during hibernation (Takamatsu et al., 1993), there is doubt as to whether this is due to low Tb. Now we show that HP production and levels are circannually downregulated even in animals with a high Tb in the warmth and that a decrease in HP in the blood is triggered prior to the onset of hibernation and sustained throughout the hibernation season despite periodic rewarming of the animal. This clearly indicates that the downregulation of HP is under control of an endogenous circannual rhythm and is not due to low Tb. This finding provides a unique molecular tool for defining the timing and period of an endogenous hibernation season under warm conditions without the animal being subjected to a low Tb in the cold and thus may allow us to overcome the difficulty in clarifying Tb-independent adaptation mechanisms for hibernation.

Despite a marked decrease in HP in the blood during the hibernation season, HP in CSF increases dramatically. Immunohistochemical signals of HP are detected and intensified during hibernation in choroidal epithelial cells,

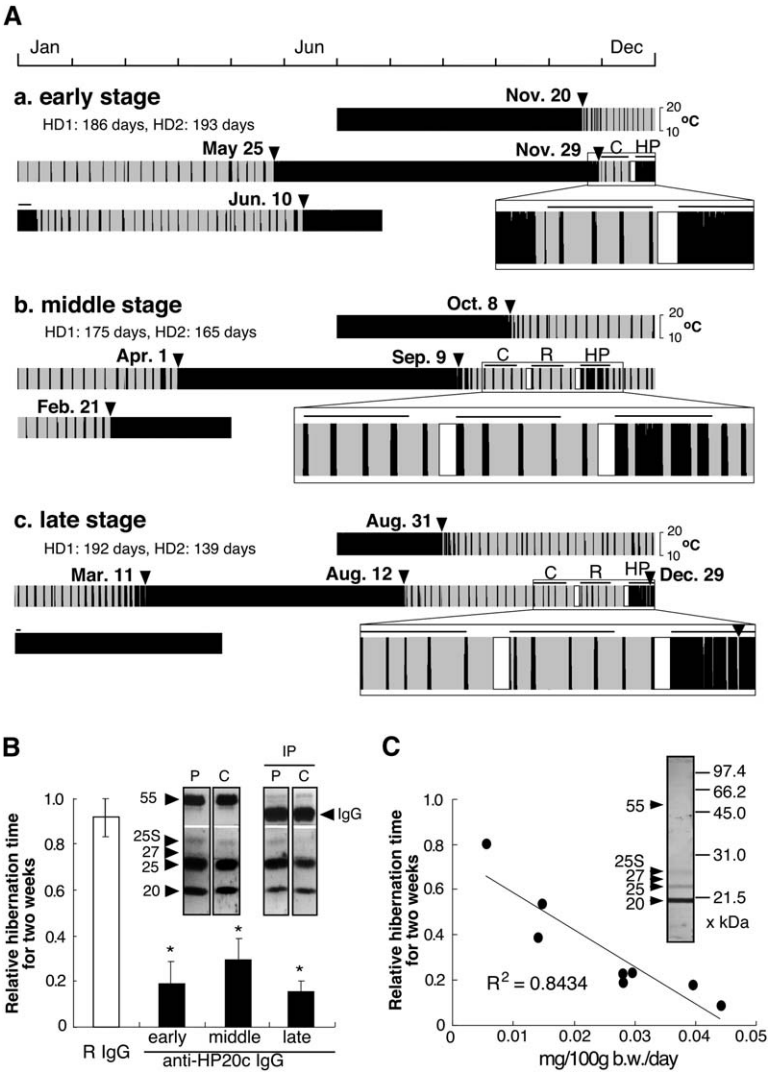

Figure 6. Inhibition of Hibernation by Polyclonal Anti-HP20c Antibody

(A) Effects of the antibody on hibernation at the early (a), middle (b), and late (c) stages in Tb-ms recordings for about 2 years in three independent animals. After determining individual hibernation duration (between the first and second arrowheads with date: HD1), the antibodies (preimmune and anti-HP20c IgGs) were sequentially administered into lateral ventricles using a 2 week-function osmotic pump implanted at each stage (between the third and fourth arrowheads with date: HD2). Hibernation time during 2 week period (each line above Tb-ms recordings) before (control: C) and during administration of PBS containing preimmune (R) and anti-HP20c (HP) IgG of 0.045 mg/100 g body weight/day was used for analysis in (B). At the early stage, however, administration of preimmune IgG could not be attempted since there was not enough time within the limited period of this stage. After each implantation, animals were kept warm (23°C, 12 hr LD cycle) for 3 days (open column) in order to ensure sufficient administration at high Tb. Tb-ms recordings enclosed in a rectangular frame are magnified in the inset.

(B) Effects of anti-HP20c IgG at the three stages (mean ± SEM; n = 5 in each stage) were compared with those of preimmune (R) IgG (mean ± SEM; n = 10). Vertical axis: hibernation time during 2 week administration of each IgG (0.045 mg/100 g body weight/day; lines with R or HP in [A]) relative to that before administration (lines with C in [A]). *Significant difference from R IgG. Inset: Western blots of HP20, 25, 27, and 55 in plasma (P) and CSF (C), and their respective immunoprecipitates (IP) by anti-HP20c IgG (left and right panels).

(C) Dose-inhibition relationship of anti-HP20c IgG in hibernation stages 3 and 4. Vertical and horizontal axes: relative hibernation time as shown in (B) and administration doses. R^2: correlation coefficient. Western blots for testing reactivity of the IgG to proteins in CSF (Inset) shows major and minor reactivities to HP20 and HP25 (HP25S), respectively. Left and right numbers: positions of each HP component and molecular marker.

although *HP* mRNA expression is not detected. This contrary regulation of HP in the blood and CSF suggests active transport mechanisms in the choroidal epithelium of the blood-CSF barrier, the major producer of CSF. This proposed transport to the brain coupled with dissociation of HP complex to HP20c and HP55 could indicate an activation step, which would suggest a difference from the transport of hormones such as insulin-like growth factors and leptin across the blood-CSF barrier in nonhibernators (Chodobski and Szmydynger-Chodobska, 2001). HP20c dissociated from HP55 in CSF may be an active form and function in the brain since HP55 is known to be a protease inhibitor (Kondo and Kondo, 1996) and a member of the serpin superfamily (Kondo and Kondo, 1992a), which includes proteins that bind hormones such as thyroxine (Flink et al., 1986) and corticosteroids (Hammond et al., 1987). Thus, we propose that, under control of a circannual rhythm, the choroidal epithelium acts in the transport and activation of HP in the brain. This system may play a key role in the hormonal signaling of HP for regulating brain functions for hibernation.

There is little possibility that brain HP20c is directly involved in thermoregulation during hibernation since Tb periodically increases by self-rewarming despite a sustained increase in HP20c in the brain throughout the hibernation season (Figure 4A). This is in agreement with the fact that in constant warmth, animals with increasing HP20c in CSF fail to lower their Tb toward the ambient temperature.

Instead, HP20c may be involved in long-term development of physiological capacity in cells and organs for tolerating severe hibernation states, such as low Tb and ischemia, throughout a hibernation season. In support of this, blocking brain HP20c activity using a specific antibody decreases in a dose-dependent manner the hibernation time during which a low Tb is tolerated, and hibernation is even terminated early in a few animals. A developed capacity for tolerance has been observed in previous in vitro studies. In chipmunk cardiac muscles, inactivation of calcium ion channels and enhancement of the ability of internal stores to take up calcium ions (Kondo and Shibata, 1984; Kondo, 1986a, 1986b, 1988) were observed only during the hibernation season, independently of Tb (Kondo, 1987; Kondo and Kondo, 1992b). These changes could prevent excessive cytosolic calcium accumulation induced by lowering Tb during hibernation that might result in cardiac cell death (Hochachka, 1986; Kondo, 1986a, 1988). Tolerance to low Tb (Pakhotin et al., 1990) and to hypoxia and aglycemia (Frerichs and Hallenbeck, 1998) has been shown in hippocampal and septal slices from hibernating ground squirrels.

Interestingly, the function of proteins homologous to HP20c in their primary and/or higher structures, such as the complement protein C1q, precerebellin, and adiponectin (Kishore and Reid, 1999) could support a role for HP in developing tolerance to the extreme physiological conditions of hibernation. Adiponectin and C1q are known to facilitate glucose uptake and fatty acid oxidation (Fruebis et al., 2001; Yamauchi et al., 2002) and to play a role in innate immunity (Kishore and Reid, 1999) in mammals. A precerebellin-like protein has been reported as an acute phase protein in fish that functions in a variety of biological defense-related activities such as repair of tissue damage, killing of microbes and other potential pathogens, and restoration of the healthy state (Bayne and Gerwick, 2001). Although the functions of HP remain to be clarified, these observations lead us to propose the involvement of HP20c in the regulation of energy metabolism and/or biological defenses, which are considered to be crucial for adapting to severe physiological states during hibernation.

Circannual HP regulation independent of Tb and environmental changes clearly indicates the existence of an internal system governing adaptation to the physiological stresses of hibernation. It is reasonable to propose that a circannual rhythm generator, probably in the brain, outputs a signal to the periphery, which downregulates HP complex in the liver and simultaneously facilitates its transport in the choroid plexus throughout the hibernation season (Figure 7). Through such crosstalk between central and peripheral organs, HP20c may modulate brain function for tolerating severe hibernating states, suggesting that hibernation results from hormonal modulations of cells and organs rather than innate characteristics specific to hibernators. Interestingly, a circannual timing mechanism may be insensitive to low Tb and aging because a circannual HP rhythm is constantly generated independently of Tb changes throughout a long life span.

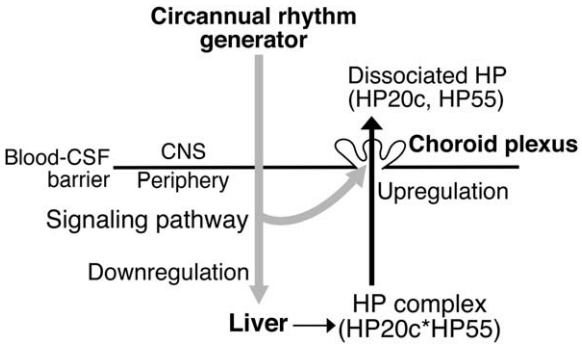

Figure 7. Proposed Schema of Control System for Hibernation

Gray arrow indicates the predicted signaling pathway from center to periphery. HP20c*HP55 and Dissociated HP20c indicate inactivated and activated HP, respectively. See text for details.

As the circannual regulation system proposed in Figure 7 would operate even under homeothermic states, this system could be applied to seasonal phenomena in nonhibernators that play a central role in controlling a wide variety of physiological events, such as reproduction in seasonally breeding mammals and migration in birds. Endogenous circannual timing mechanisms constrain a phase of depressed reproduction in seasonal reproductive cycles (Bronson, 1989) and control seasonal variations in migratory behavior such as the onset and end of migration and the duration of migratory restlessness (Gwinner, 1996). Even in humans, seasonal rhythms have been observed, as seasonal affective disorder exhibiting annually recurrent depressions (Aschoff, 1981; Rosenthal et al., 1984), which is characterized by hypersomnia, hyperphagia, and weight gain, similar to those seen in seasonal hibernation. Thus, these seasonal changes are likely to be controlled by a common circannual signal, although exactly what it is remains unknown. A candidate for this signal might be identified by exploring factors that regulate HP in the liver and choroid plexus as shown in Figure 7. The identification of a signal and signaling pathway in future studies may help us to develop a molecular approach to mechanisms governing circannual rhythms.

In conclusion, we have provided evidence for a candidate hormone that may modulate brain functions in order to develop a capacity for tolerating low Tb during hibernation. Future studies infusing active HP20c into the brain will be required to assess whether HP is sufficient for the induction of hibernation. The present findings will stimulate future experiments on HP addressing the control of hibernation and potential pharmacological applications in humans to the prevention of lethal diseases such as hypothermia, ischemia, muscle atrophy, bacterial infection, and tumorigenesis, which has been observed during hibernation in hibernators. These studies may further stimulate the exploration of new techniques for cryosurgery of the heart and brain, as well as the development of hypothermia treatment that is effective for preventing brain

ischemic damage (Hypothermia After Cardiac Arrest Study Group, 2002).

EXPERIMENTAL PROCEDURES

Animals and Blood Collection

Male chipmunks (*Tamias sibiricus*) born within a year of one another (Tokyo Experimental Animals Co., Japan) were kept under constant conditions of 23°C and a 12 hr light-dark (LD) photoperiod and given a standard rat chow diet and water ad libitum. The animals were used in experiments after at least 6 months in captivity under laboratory conditions. The animals were divided into two groups, one kept under constant conditions of cold (5°C) and darkness and another under conditions of warmth (23°C) and a 12 hr LD cycle. Blood samples were collected monthly from a femoral vein. This study, whose aim was to obtain initial conclusive evidence for characterizing the relationship between a free-running circannual rhythm and HP regulation in chipmunks, was performed in accordance with the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science) and was approved by our institute's committee. Experiments that continue throughout the entire life of an animal are essential for examining the association between a free-running circannual rhythm of hibernation with HP and a lack of hibernation in animals without circannual HP regulation.

Surface Tb Measurement in the Cold and Detection of Torpor in the Warmth

Animals kept in individual plastic cages (H:W:D, 20:25:40 cm), the top of which was covered with stainless steel lattice, were placed on an animal rack in a cold (5°C), dark room. The surface body temperature (Tb) was measured at 30 min intervals using a computer-controlled infrared Thermal Video System (TVS-2300ST) (Nippon Avionics Co. Ltd.; Figures 1A and 1B). Dead spaces in the cages that were not measured were masked using aluminum plates. This is the best method for long-term, noninvasive measurements and was originally developed by us for solving the serious problems which disturb hibernation, such as invasive treatments for measuring Tb. By analyzing the maximum surface Tb (Tb-ms) detected in each thermal image, the onset, termination, and duration of hibernation were determined within an accuracy of 0.5 hr (Figure 1C). Fluctuations in Tb-ms during an active state are due to continual changes in the position of an animal reflected by high locomotor activity. A zone defined as the area of Tb-ms between 10°C and 20°C (red frame in Figure 1C) was used for representing Tb changes in Figures 4A and 6A, and the zone accurately distinguishes between hibernation and arousal phases and can be used to calculate the actual hibernation time (time during low Tb: gray areas). This accurate calculation of time during low Tb is essential for evaluating the effects of an antibody on hibernation. On the other hand, animals in the other group were kept at 23°C to prevent hibernation since keeping an animal constantly warm at 22°C–23°C has routinely been carried out to prevent hibernation. However, even under these conditions, the Tb of a few animals decreased to near room temperature, and the animals had greatly reduced locomotor activity (shallow torpor) during the hibernation season. The thermal video system described above was not suitable for detecting such a mild decrease in Tb due to the inaccuracy of the measurements because of the little thermal difference between the surface Tb and the high background temperature of 23°C. However, since the shallow torpor with a mild decrease in Tb was easily detected by markedly depressed respiration and locomotor activity due to the lower Tb, the animals were checked visually and tactually every morning and evening. Animals exhibiting shallow torpor were excluded from the present experiments.

Western Blot Analysis

Proteins corresponding to 10–25 nl of plasma and 1–2 μl of CSF per lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide) and transferred onto a PVDF membrane using an electroblotting apparatus. Antisera against HP20, 25, 27, and 55 from rabbits were used as primary antibodies. Detection was performed using an ECL Western Blotting Detection System (Amersham). Signals were visualized using X-ray film (Kodak X-Omat). Images were generated by scanning the photograph of the developed membrane with Adobe Photoshop software. The densities of the signals for HP20, 25, and 27, and the background of each lane were measured with NIH image software, and each specific signal was obtained by subtracting the background. Levels of HP complex (HPc) in the blood and HP in CSF were determined by measuring total densities for HP20, 25, and 27.

Northern Blot Analysis

Total RNA was prepared from the liver of chipmunks by the guanidium isothiocyanate method (Sambrook et al., 1989). Poly(A)$^+$-RNA was purified using Oligotex-dT30 (Takara Shuzo). Liver poly(A)$^+$-RNA was fractionated by electrophoresis on 1.0% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and fixed by Stratalinker (Stratagene). The membrane was probed with the chipmunk *HP25* cDNA and then reprobed with the chipmunk albumin cDNA.

Immunohistochemistry

Animals were anesthetized with pentobarbital and then transcardially perfused with cold periodate-lysin-0.4% paraformaldehyde (PLP) solution. The brain was cut into 2 mm slices and equilibrated with PLP solution. These slices were cut into 6 μm sections on a freezing microtome. Cryosections were stained using affinity-purified anti-HP20c polyclonal antibody from rabbit followed by fluorescein isothiocyanate-conjugated goat second antibody (Jackson). Control sections were stained without the primary antibody.

Real-Time RT-PCR Analysis

Total RNA was isolated from several brain regions and liver using an RNeasy Mini Kit (Qiagen). Real-time RT-PCR was performed with a TaqMan OneStep RT-PCR Master Mix Reagent kit and ABI PRISM 7000 (Applied Biosystems) using 50 ng of DNase-treated total RNA. Gene-specific primers and TaqMan probes for chipmunk HP25 were as follows: 5′-GTCTGAATGCCTGCACAAAGAG-3′ (forward primer), 5′-GATGGACAGGACCAAAATCCA-3′ (reverse primer), 5′-FAM-TGGGAGCTGCTGGC-MGB-3′ (TaqMan). For chipmunk β-actin, they were as follows: 5′-ACTGGGACGACATGGAGAAAA-3′ (forward primer), 5′-CCACACGCAGCTCGTTGTA-3′ (reverse primer), 5′-VIC-TTGGCACCACACTTT-MGB-3′ (TaqMan). RNA from liver was stepwise diluted and used to generate standard curves for each primer system.

CSF Collection and Analysis of HP Changes

Animals were anesthetized with pentobarbital (0.1 mg/100 g body weight; Dainippon Pharmaceutical Co.) and ketamine (1.5 mg/100 g body weight; Sankyo & Co.), and then their heads were fixed on a stereotaxic apparatus (SR-5N: Narishige). After exposing cranial bones around the bregma, a cannula for collecting CSF was inserted into the lateral ventricles and fixed to cranial bone using dental cement. After this procedure, the animals were kept at 5°C in darkness or at 23°C with 12 hr LD cycles for a week. CSF and blood were then collected and stored at −80°C until use. For the statistical analysis of HP levels in the blood and CSF during the hibernation season, active and hibernating states in each animal were divided into five stages (Figure 1D) for the following reasons: the periods of active and hibernating states differed individually (Figure 2A), HP levels in the blood and CSF collected monthly changed during the hibernation period (Figure 4A), and the hibernation season was approximately 5 months in the major hibernating animals used. HP levels at each stage of the hibernating state were compared with those at the third stage (3) of the active state where the blood and CSF HP levels reached constant levels (Figure 4B).

Size Exclusion Chromatography Analysis

Plasma (5 μl) and CSF (60 μl) were separated on a Protein Pak 300 column (0.6 × 60 cm, Waters Corp.) using phosphate-buffered saline (pH 6.9) at a flow rate of 0.5 ml/min. The fractions of plasma and CSF collected every 0.5 min were sequentially numbered from 15 min after loading samples, and 10 (plasma) and 200 (CSF) μl of each fraction were analyzed by Western blotting.

Administration of Antibody and Evaluation of Its Effects

The anesthesia and cannula implantation procedures used for administration were the same as those described in "CSF Collection and Analysis of HP Changes." Immunoglobulin (IgG) was purified from rabbit serum before and after immunization with HP20c using a protein G immobilized affinity column, diluted in phosphate buffered saline (PBS; pH 7.4) and then administered into the lateral ventricles through the implanted cannula connected to a Model 2002 Alzet osmotic minipump (DURECT Co.) that had been implanted intraperitoneally. The pump functions for 2 weeks at an outflow rate of 0.5 μl/hr at normal Tb. In order to evaluate quantitatively the inhibitory effects of anti-HP20c IgG on hibernation, hibernation was defined as the time during Tb-ms below 10°C ("Hibernation time" in Figure 1C), and total hibernation time during 2 week administration of PBS containing IgGs was compared to that during 2 weeks before administration. A comparison was performed at the three stages redefined in Figure 1D for distinguishing major changes in HP content in CSF during the hibernation season (Figure 4A).

Immunoprecipitation of HP20c in the Blood and CSF

Plasma and CSF were immunoprecipitated with the anti-HP20c IgG and anti-HP27 IgG. Anti-HP27 IgG was isolated from rabbit serum after immunization with HP27, which was purified from plasma under denaturing and reducing conditions as described previously (Kondo and Kondo, 1992a). Anti-HP20c IgG immunoprecipitated HP20c in plasma but did not coprecipitate HP55, as shown in Figure 6B inset (thus the antibody interferes with the interaction between HP20c and HP55), while anti-HP27 IgG coimmunoprecipitated HP20c with HP55 (HPc) in plasma. The immunoprecipitates were subjected to Western blotting.

Statistical Analysis

The levels of HP in plasma and CSF and the inhibitory effects of intraventricular administration of antibody on hibernation were compared using paired and unpaired t tests. A difference was considered significant if $p < 0.05$.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/cgi/content/full/125/1/161/DC1/.

ACKNOWLEDGMENTS

We thank A. Narita and C. Suzuki of KAST for collecting the blood and performing the Western blot analysis and K. Saito, M. Matsuo, and S. Kamijo of Mitsubishi Kagaku Institute of Life Sciences for maintaining and feeding the chipmunks. This work was supported, in part, by the National Space Development Agency of Japan.

Received: June 29, 2005
Revised: October 11, 2005
Accepted: March 13, 2006
Published: April 6, 2006

REFERENCES

Amorese, D.A., Swan, H., and Bamburg, J.R. (1982). Extracts from the brains of hibernating and alert ground squirrels: effects on cells in culture. Proc. Natl. Acad. Sci. USA *79*, 6375–6379.

Aschoff, J. (1981). Annual rhythms in man. In Handbook of Behavioral Neurobiology, 4, J. Aschoff, ed. (New York: Plenum Press), pp. 475–487.

Bayne, C.J., and Gerwick, L. (2001). The acute phase response and innate immunity of fish. Dev. Comp. Immunol. *25*, 725–743.

Bronson, F.H. (1989). Mammalian Reproduction Biology (Chicago: University of Chicago Press).

Chodobski, A., and Szmydynger-Chodobska, J. (2001). Choroid plexus: target for polypeptides and site of their synthesis. Microsc. Res. Tech. *52*, 65–82.

Flink, I.K., Bailey, T.J., Gustafson, B.E., Markham, B.E., and Morkin, E. (1986). Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to the serine antiproteases. Proc. Natl. Acad. Sci. USA *83*, 7708–7712.

Frerichs, K.U., and Hallenbeck, J.M. (1998). Hibernation in ground squirrels induces state and species-specific tolerance to hypoxia and aglycemia: an in vitro study in hippocampal slices. J. Cereb. Blood Flow Metab. *18*, 168–175.

Fruebis, J., Tsao, T.-S., Javorshchi, S., Ebbets-Reed, D., Erickson, M.R.S., Yen, F.T., Bihain, B.E., and Lodish, H.F. (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc. Natl. Acad. Sci. USA *98*, 2005–2010.

Gwinner, E. (1996). Circadian and circannual programmes in avian migration. J. Exp. Biol. *199*, 39–48.

Hammond, G.L., Smith, C.L., Goping, I.S., Underhill, D.A., Harley, M.J., Reventos, J., Musto, N.A., Gunsalus, G.L., and Bardin, C.W. (1987). Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs. Proc. Natl. Acad. Sci. USA *84*, 5153–5157.

Harlow, H.J., Lohuis, T., Beck, T.D.I., and Iaizzo, P.A. (2001). Muscle strength in overwintering bears. Nature *409*, 997.

Hochachka, P.W. (1986). Defense strategies against hypoxia and hypothermia. Science *231*, 234–241.

Hypothermia After Cardiac Arrest Study Group. (2002). Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N. Engl. J. Med. *346*, 549–556.

Kemper, G.B., and Ruben, R.L. (1982). Effect of 7,12-dimethylbenz(a)anthracene on the integument of the hibernating and nonhibernating 13-lined ground squirrel. Comp. Biochem. Physiol. *73C*, 445–450.

Kishore, U.K., and Reid, B.B. (1999). Modular organization of proteins containing C1q-like globular domain. Immunopharmacology *42*, 15–21.

Kojima, M., Shiba, T., Kondo, N., and Takamatsu, N. (2001). The tree squirrel *HP-25* gene is a pseudogene. Eur. J. Biochem. *268*, 5997–6002.

Kondo, N. (1986a). Excitation-contraction coupling in myocardium of nonhibernating and hibernating chipmunks: effects of isoprenaline, a high calcium medium and ryanodine. Circ. Res. *59*, 221–228.

Kondo, N. (1986b). Excitation-contraction coupling in the myocardium of hibernating chipmunks. Experientia *42*, 1220–1222.

Kondo, N. (1987). Identification of a pre-hibernating state in myocardium from nonhibernating chipmunks. Experientia *43*, 873–875.

Kondo, N. (1988). Comparison between effects of caffeine and ryanodine on electromechanical coupling in myocardium of hibernating chipmunks: role of internal Ca stores. Br. J. Pharmacol. *95*, 1287–1291.

Kondo, N., and Shibata, S. (1984). Calcium source for excitation-contraction coupling in myocardium of nonhibernating and hibernating chipmunks. Science *225*, 641–643.

Kondo, N., and Kondo, J. (1992a). Identification of novel blood proteins specific for mammalian hibernation. J. Biol. Chem. *267*, 473–478.

Kondo, N., and Kondo, J. (1992b). Identification of novel types of protein specific for mammalian hibernation with circannual rhythm. In Circadian Clocks from Cell to Human, T. Hiroshige and K. Honma, eds. (Sapporo, Japan: Hokkaido University Press), pp. 89–96.

Kondo, N., and Kondo, J. (1993). Identification and characterization of novel types of plasma protein specific for hibernation in rodents. In Life in the Cold: Ecological, Physiological and Molecular Mechanisms, C. Carey, G.L. Florant, B.A. Wunder, and B. Horwitz, eds. (Boulder, CO: Westview Press Inc.), pp. 467–473.

Kondo, J., and Kondo, N. (1996). Structural aspects of complex of hibernation-specific proteins. In Adaptation to The Cold, F. Geiser, A.J. Hulbert, and S.C. Nicol, eds. (Armidale, Australia: University of New England Press), pp. 351–355.

Pakhotin, P.I., Belousov, A.B., and Otmakhov, N.A. (1990). Functional stability of the brain slices of ground squirrels, *Citellus undulatus*, kept in conditions of prolonged deep periodic hypothermia: electrophysiological criteria. Neuroscience *38*, 591–598.

Pengelley, E.T., and Asmundson, S.J. (1974). Circannual rhythmicity. In Circannual Clock: Annual Biological Rhythms, E.T. Pengelley, ed. (San Francisco: Academic Press), pp. 95–160.

Rosenthal, N.E., Sack, D.A., Gillin, J.C., Lewy, A.J., Goodwin, F.K., Davenport, Y., Mueller, P.S., Newsome, D.A., and Wehr, T.A. (1984). Seasonal affective disorder. Arch. Gen. Psychiatry *41*, 72–80.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sharapov, V.M. (1983). Influence of animal hibernation on the development of mycoses. Mycopathologia *84*, 77–80.

Srere, H.K., Wang, L.C.H., and Martin, S.L. (1992). Central role for differential gene expression in mammalian hibernation. Proc. Natl. Acad. Sci. USA *89*, 7119–7123.

Takamatsu, N., Ohba, K.-I., Kondo, J., Kondo, N., and Shiba, T. (1993). Hibernation-associated gene regulation of plasma proteins with a collagen-like domain in mammalian hibernators. Mol. Cell. Biol. *13*, 1516–1521.

Takamatsu, N., Kojima, M., Taniyama, M., Ohba, K.-I., Uematsu, T., Segawa, C., Tsutou, S., Watanabe, M., Kondo, J., Kondo, N., et al. (1997). Expression of multiple α1-antitrypsin-like genes in hibernating species of the squirrel family. Gene *204*, 127–132.

Wang, L.C.H. (1982). Hibernation and the endocrine. In Hibernation and Torpor in Mammals and Birds, C.P. Lyman, J.S. Willis, A. Malan, and L.C.H. Wang, eds. (London: Academic Press), pp. 206–236.

Wang, L.C.H. (1988). Mammalian hibernation: An escape from the cold. In Advances in Comparative and Environmental Physiology, *Vol. 2*, R. Gilles, ed. (Berlin: Springer-Verlag), pp. 1–45.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., et al. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat. Med. *8*, 1288–1295.