IMMUNOHISTOCHEMICAL AND MORPHOLOGICAL CHANGES IN CHIPMUNK CAROTID BODY DURING HIBERNATION

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Abstract: Mammalian hibernators experience drastic changes in vital signs such as body temperature, respiratory rate, and heart rate during hibernation because of periodic arousals during which vital signs return to non-hibernating levels. The carotid body, an arterial chemoreceptor organ regulating respiration, contains several neuroactive substances. However, little is known about changes of neuroactive substances in the carotid body during hibernation. Immunohistochemical study using antibodies against neuroactive substances, namely tyrosine hydroxylase (TH), methionine-enkephalin (Met-Enk), and gamma-aminobutyric acid (GABA) showed that immunoreactivities for TH, Met-Enk and GABA in type I cells of carotid bodies in hibernating animals increased in comparison with those in non-hibernating ones. Furthermore, we monitored vital signs of chipmunks during arousal from hibernation, and found that the heart rate increased exponentially and the respiratory rate increased linearly as body temperature gradually increased. Subsequent examination of TH-immunoreactivity in carotid bodies during arousal showed that the TH-immunoreactivity decreased during the course of arousal from hibernation. The type I cells enlarged in size during hibernation, but the sinusoidal capillaries around them did not. These results suggest that several neuroactive substances in type I cells play a significant role in the regulation of chemoreception during the hibernating physiological state.

Key words: TH, neuroactive substance, hibernation, carotid body, immunohistochemistry, chipmunk

Introduction

Mammalian hibernation is characterized by drastic reductions in body temperature, respiratory rate, and heart rate. It has been suggested that the neuroendocrine system of the brain plays an important role in the regulation of hibernation. Numerous investigators have reported seasonal changes in several neuroactive substances in the central nervous system of hibernators, and their works have revealed that these neuroactive substances participate in the regulation of the entrance, maintenance and arousal phases of hibernation[1-5].

Hypothermia during mammalian hibernation is not continuous, but is interrupted periodically by arousals that return the animal to euthermy[6-8].

The elevation of the body temperature is accompanied by large changes in respiratory rate and heart rate. These drastic physiological changes may be regulated not only by the brainstem, but also by the chemoreceptor cells of the carotid body, which is an arterial chemosensory organ regulating respiration. Carotid body chemoreceptors, which are sensitive to hypoxia, low pH and hypercapnea, are located at the bifurcation of the common carotid artery, and they consist of two different types of cells and vasculature. The so-called type I cells contain a large number of dense-cored vesicles, and type II cells are sustentacular. Furthermore, the carotid body contains sinusoidal capillaries that are in close contact with type I and type II cells. Numerous studies have shown the presence of several neuroactive substances in type I cells[9-13]. Moreover, remarkable changes in these neuroactive substances have been reported to occur under hypoxic conditions[14-18].

However, less attention has been paid to the functional and morphological changes that occur in the carotid body during hibernation. In the present study, we aimed to examine whether or not the distribution of several neuroactive substances (tyrosine hydroxylase (TH), methionine-enkephalin (Met-Enk), and gamma-aminobutyric acid (GABA)) in type I cells changes between hibernating and non-hibernating conditions. We compared the immunoreactivity against three neuroactive substances of the cells in the carotid body of hibernating and non-hibernating chipmunks. In addition, we measured sizes of type I cells and sinusoidal capillaries in the carotid bodies of the same hibernating and non-hibernating chipmunks.

Furthermore, we measured vital signs, such as respiratory rate, heart rate, and rectal and surface temperatures, to verify the drastic changes that occur during arousal from hibernation, and then examined the immunoreactivity for TH in chipmunk carotid bodies in hibernating state, one hour and two hours after onset of arousal from hibernation.

Materials and Methods

Materials

Twenty-five adult male chipmunks (Tamias sibiricus) were used in this study. They were divided into two groups: fifteen hibernating and ten non-hibernating chipmunks. The hibernating group was housed separately with free access to food and water, and exposed to a 12 h light (600 lx)/12 h dark photoperiod. The room temperature was gradually reduced from 20°C to 8°C over a 2-month period, and kept at 8±2°C during the hibernation season. The hibernation state was verified daily by checking the excrements. Hibernating chipmunks were those who had completed at least two weeks after the first verification of the hibernation while still hibernating. The hibernation was tested by the response of the chipmunk to a mechanical stimulus. The non-hibernating group was kept at room temperature throughout the study period. Five animals of 15 hibernating and one of 10 non-hibernating chipmunks were used for measurements of respiratory rate, heart rate, rectal temperature (Tr), and surface temperature at the nose (Ts). In above animals, the one non-hibernating animal (arousal state) was used under anesthetization. A mean body weight of 6 animals used for physiological measurements was 80 g.

Physiological measurements

The respiratory rate during arousal from hibernation (i.e., during rewarming) was measured by attaching a strain gauge (D-FAE-5-512 T11, Minebea Co., Japan) on the chest of hibernating chipmunks. This strain gauge was connected to an amplifier (AS1201, NEC, Japan). The heart rate was measured in the form of an electrocardiogram. Rectal temperature was measured with a digital thermometer (SK-1250MC, Sato Keiryoki, Japan), and the surface temperature was measured indirectly at the animal's nose by an infrared-beam-type thermometer (Circle Thermo SK-8100, Sato Keiryoki, Japan). These vital signs were recorded on both a DAT recorder (PC208Ax, Sony, Japan) and

a pen recorder (Omniace RT330, NEC, Japan).

Tissue preparation

Chipmunks in non-hibernating and in the level of arousal from hibernation were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), while hibernating animals were under low-temperature anesthesia. For fixation, these animals were perfused through the heart with ice-cold 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) at a constant flow rate. Carotid bodies of 25 chipmunks were subjected to immunohistochemical investigation using each specific antibody against TH, Met-Enk, and GABA. The left and right carotid bodies and adjacent tissues containing the common carotid artery and its branches were dissected out from the fixed animals under a dissecting microscope. These tissues were post-fixed in the same fixative for 8-12 h at 4°C. After being washed briefly in 1 mM phosphate buffer saline (PBS, pH 7.4), the carotid bodies were removed from adjacent tissues and rinsed for 12-24 h in 10 mM PBS containing 30% sucrose. Prepared carotid bodies were then frozen in dry ice and sectioned at 10 μm on a cryostat (Histostat Microtome 2200, Meiwa Shoji, Japan), and mounted on 3-aminopropyltriethoxy-silane-coated slides (Matsunami, Japan).

Immunoreactivity

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase method. First, endogenous peroxidase activity was quenched by treating the sections with 0.3% hydrogen peroxide in 0.2% Triton-X in 10 mM PBS (PBS-T) for 30 min at room temperature. After being rinsed three times in PBS-T, the sections were incubated at 4°C for 18-24 h with primary antisera to TH (1 : 1000 ; Chemicon, USA), Met-Enk (1 : 2000 ; Chemicon), or GABA (1 : 400 ; Chemicon), and then diluted in a solution of 1% bovine serum albumin in 10 mM PBS-T (pH 7.4) at room temperature. After rinsing with PBS-T, the sections were incubated with goat anti-rabbit IgG (1 : 200 ; Jackson, USA) in 10 mM PBS-T (pH 7.4) for 2 h at room temperature. Next they were rinsed three times in PBS-T, incubated with rabbit peroxidase-antiperoxidase complex (1 : 200 ; Dako, Denmark) in 10 mM PBS-T for 2 h at room temperature. Then the sections were rinsed three times with 50 mM Tris-HCl buffer at pH 7.5. The peroxide activity was visualized with 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (Sigma, USA) in 50 mM Tris HCl buffer (pH 7.5) containing 0.01% hydrogen peroxide for 10 min at room temperature[19]. The sections were dehydrated through a graded ethanol series (50-100%), cleared with xylen and then mounted in Permount (Fisher, USA).

Measurements

Sizes of cells and capillaries were measured on micrographs at a final magnification of ×1,220 ; the magnification was calculated using a calibration grid (Absolute Digimatic, Mitutoyo, Japan) on 31 cells of 6 sections taken from 3 non-hibernating carotid bodies and 32 cells of 8 sections taken from 4 hibernating carotid bodies. As the profiles of cells in sectioned carotid bodies are, in most cases, oval in shape, measurement of the long axis of this profile gave an overestimation of the diameter. For the size of sinusoidal capillaries in the carotid body, the inner diameter of the vessel was measured on 76 sinusoidal capillaries in 6 sections taken from 3 non-hibernating and 73 sinusoidal capillaries in 8 sections taken from 4 hibernating carotid bodies. Results were expressed as mean± standard deviation (SD). Morphometric analysis of the immunoreactive cells was performed using an image analysis system (VIDAS ; Zeiss, Germany). Immunolabeled cells were selected in binary images. The measuring fields per section were randomly selected from the parenchyma of the carotid body.

Results

1) Physiological results

In non-hibernating animals, the respiratory and

Table 1 Measured respiratory rate, heart rate and rectal (Tr) and surface (Ts) temperatures at the nose at the beginning and the final of arousal from hibernation in chipmunks.

state		*Tr* (℃)	*Ts* (℃)	Respiratory rate (breaths/min)	Heart rate beats/min
arousal	beginning	7.0±0.1[2)]	14.6±1.6[2)]	24.8±8.0[2)]	40.2±4.9[2)]
	final	15.6±2.1[2)]	21.1±1.3[2)]	180.0±18.3[2)]	433.8±43.2[2)]
non-hibernation		23-25[1)]	61.3[1)]	445.0[1)]	

1) Vital signs of the non-hibernating control chipmunk ($n=1$) was measured under anesthetized condition.
2) Data were expressed as mean±SD for arousal chipmunks ($n=5$).

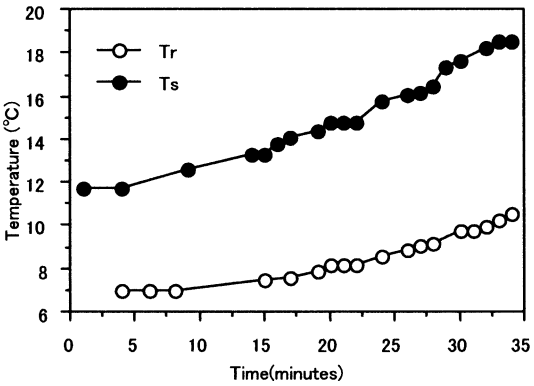

Fig. 1 Typical changes in rectal temperature (*Tr*) and surface temperature at nose (*Ts*) during arousal from hibernation. Observations began when the chipmunk was exposed to room temperature. The arousal started with an increase in *Ts*, followed by an increase in *Tr*.

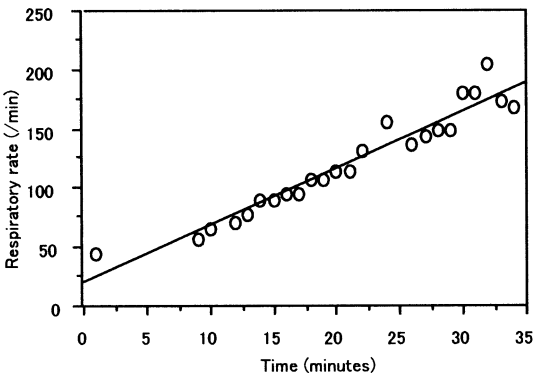

Fig. 2 Typical changes in respiration rate during arousal from hibernation. Observations began when the chipmunk was exposed to room temperature. Respiratory rate changed linearly ($Y=20.57+4.82X$, $p<0.005$).

heart rates were 61 breaths/min and 445 beats/min, respectively (Table 1). Hibernating chipmunks remained in the curled position when handled. At the beginning of arousal from hibernation, the respiratory and heart rates were below 24.8 breaths/min and 40.2 beats/min, respectively, and *Tr* and *Ts* were 7.0℃ and 14.6℃, respectively (Table 1). Vital signs were measured until animals became unstable due to their moving from the curled position. The final measured values of the vital signs during the course of arousal were 180.0 breaths/min for the respiratory rate, 433.8 beats/min for the heart rate, 15.6℃ for *Tr*, and 21.1℃ for *Ts* (Table 1). During arousal from hibernation, respiratory and heart rates initially increased, and subsequently the body temperature began to rise gradually. In this process, the respiratory and heart rates sometimes became irregular and sometimes became rhythmic, and began to increase slowly and gradually. In addition, there was a difference in changes between *Tr* and *Ts* during arousal from hibernation. The lowest temperatures were 7.0℃ for *Tr* and 14.6℃ for *Ts*, and following arousal Ts increased first, followed by Tr (Fig. 1). The respiratory rate in the non-hibernating state (61.3 breaths/min) was lower than that at the end of arousal from hibernation (180 breaths/min). It is considered that the lower respiratory

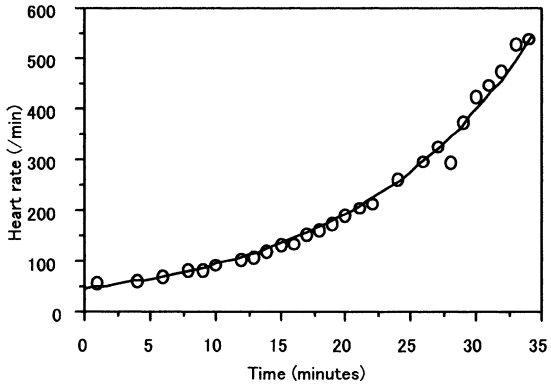

Fig. 3 Typical increase in heart rate during arousal from hibernation. Observations began when the chipmunk was exposed to room temperature. Heart rate showed an exponential increase ($Y=45.65e^{0.072*X}$, $p<0.001$). The solid line shows the best fit to the exponential function.

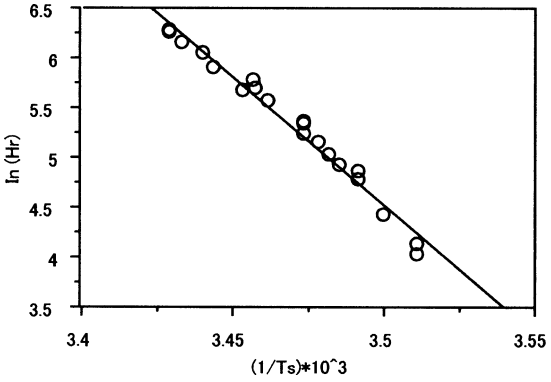

Fig. 4 Relationship between surface temperature at nose (Ts) and heart rate (Hr) of chipmunk during arousal from hibernation. The relationship between $1/Ts$ and in (Hr) showed linear. ($Y=94.53-25.71*X$; $\hat{R}2=0.98$)

rate in the non-hibernating state was due to the anesthetized condition.

The present analysis of changes in vital signs during arousal from hibernation shows that the respiratory rate increased linearly (Fig. 2) from 25 to 180 breaths/min whereas the heart rate increased exponentially (Fig. 3) from 40 to 434 breaths/min within 35-45 min, which was confirmed by our preliminary experiments[20]. The relationship between $1/Ts$ and ln (Hr) during arousal from hibernation was linear (Fig. 4).

2) Immunohistochemical results

TH immunoreactivity was observed in almost all type I cells in both non-hibernating and hibernating animals, and was more intense in hibernating animals than in non-hibernating ones (Fig. 5, 1a, b). However, this intense immunoreactivity in hibernating animals decreased during 1 or 2 hours after the onset of arousal from hibernation (Fig. 6).

For Met-Enk, intensely immunoreactive cells were distributed throughout the parenchyma of the carotid bodies. The area of highest reactivity was around the sinusoidal capillaries. The immunostaining of type I cells of hibernating animals was more intense compared with that of non-hibernating ones (Fig. 5, 2a, b). The quantitatave analysis based on the binary images of manual section using an image analysis system revealed that differences in both TH and Met-Enk-immunoreactive areas between hibernating and non-hibernating animals were statistically significant (Student's t test ; $p<0.05$), respectively. Intense GABA immunoreactivity in chipmunk carotid bodies was found to be enhanced by hibernation (Fig. 5, 3a, b).

3) General histology

The longitudinal axis of the type I cells was found to be 22.5% longer in hibernating animals (12.3 ± 1.2 μm) than in non-hibernating control animals (10.0 ± 0.8 μm) (Fig. 7). The diameter of sinusoidal capillaries around type I cells was 13% smaller in hibernating animals (6.7 ± 1.7 μm) than in non-hibernating control animals (7.7 ± 1.2 μm) (Fig. 7).

Discussion

Physiological aspects

Hypothermia during mammalian hibernation is periodically interrupted by arousals to euthermy[6,7]. The resulting elevation of body temperature is

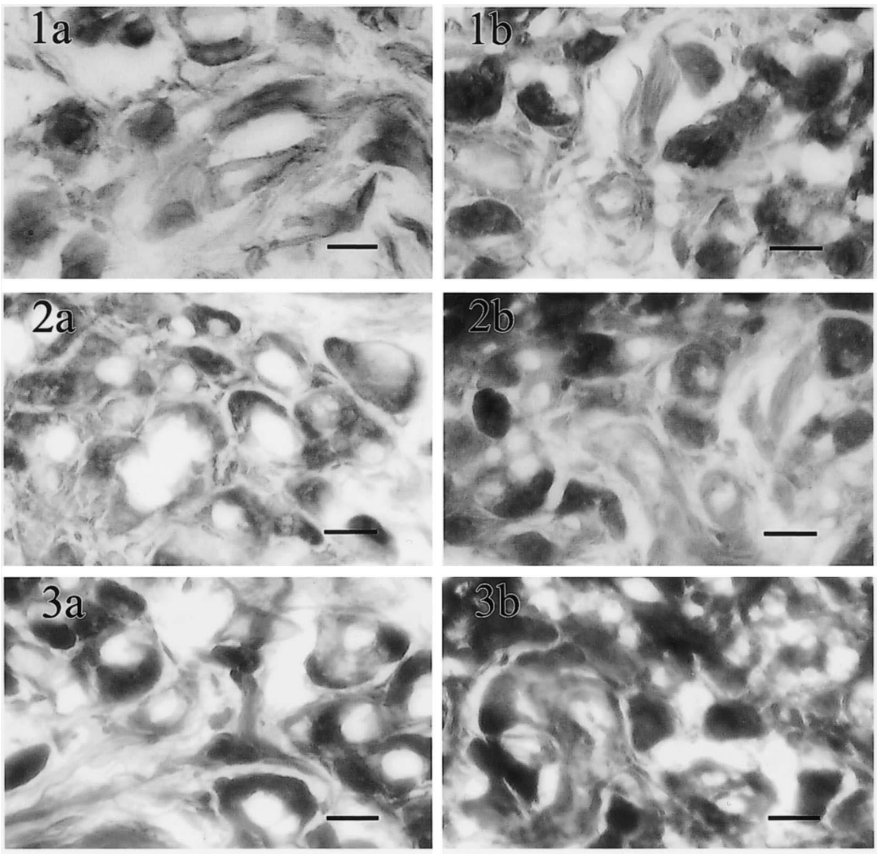

Fig. 5 TH (1), Met-Enk (2) and GABA (3) immunoreactivities in type I cells of non-hibernating (a) and hibernating (b) chipmunks. Scale bar=20 μm.

accompanied by drastic changes in respiratory and heart rates. We measured these changes in vital signs, and found that respiratory and heart rates initially increased, and subsequently the body temperature began to rise gradually. The entrance into hibernation in ground squirrels[21] and the African blue-naped mouse bird[22] begins by a drop in heart rate, followed by a gradual decline in body temperature. These data show that both the arousal from hibernation and the entrance into hibernation are started by changes in respiratory and heart rates.

Moreover, hibernating animals experience periodical arousals that are accompanied by drastic changes of respiratory rate. Our data from the chipmunk showed that during arousal from hibernation the respiratory rate increased linearly, whereas the heart rate increased exponentially within 35-45 min. Similar observations have been reported for the western jumping mouse during arousal from hibernation[23] and for the Estruscan shrew rewarming from torpor[24]. In this study on chipmunks during arousal from hibernation, the relationship between $1/Ts$ and $\ln(Hr)$ showed linear, while in our previous study on horseshoe bats there were two linear components with a break point at 17°C in the relationship[25]. These results suggest that the difference for this relationship between chipmunk and bat may be caused from the difference in amount of brown adipose tissue stored before hibernation season. Because it is known that two types of heat production are involved during arousal from

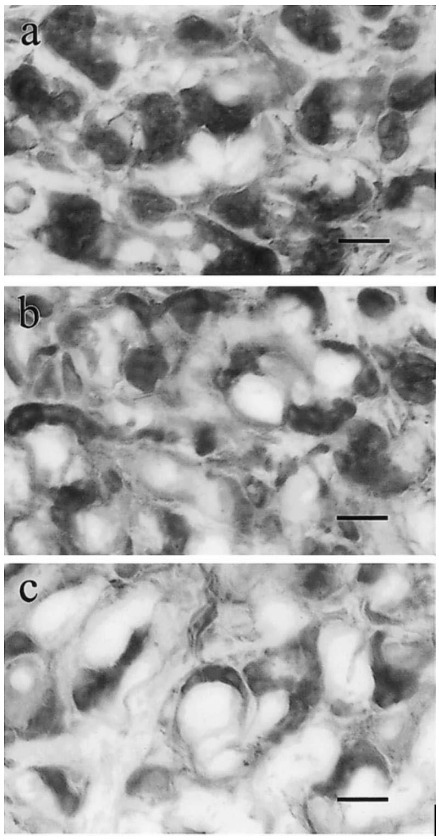

Fig. 6 Changes in TH immunoreactivity in type I cells of the carotid body during hibernation (a), 1 h (b) and 2 h after onset of arousal from hibernation. Scale bar=20 μm.

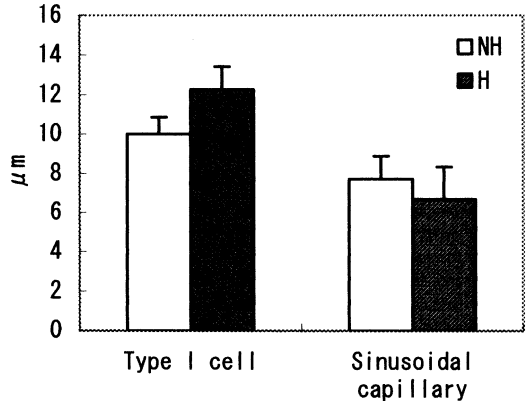

Fig. 7 Effects of hibernation (shadowed columns) on the longitudinal axis of type I cells and on the diameter of sinusoidal capillaries in carotid bodies. The "Type I cell" and "Sinusoidal capillary" referred to the length of the longitudinal axis of type I cells and the diameter of sinusoidal capillaries, respectively. The length of type I cells in hibernating chipmunks was significantly greater than that in non-hibernating animals. The diameter of sinusoidal capillaries in hibernating chipmunks was less than that in non-hibernating animals. Data are expressed as the mean±SD (type I cell: $n=32$ for hibernating and $n=31$ for control, $p<0.001$; sinusoidal capillary: $n=73$ for hibernating and $n=76$ for control, $p<0.001$).

hibernation: shivering thermogenesis from the skeletal muscle and non-shivering thermogenesis from the brown adipose tissue, and the proportional contribution of shivering and nonshivering is different among species.

Immunohistochemical aspects

Previous studies have demonstrated the presence of various neuroactive substances in the type I cells of the carotid body[9-12,26]. Hibernation is considered to be a severe hypoxic condition because hypothermia causes a high oxygen affinity of hemoglobin. In addition, the cerebral blood flow in hibernating ground squirrels is approximately one -ninth of that in active control animals[21]. Many studies have shown that hypoxia induces the change of several neurotransmitters and putative neuromodulators in type I cells[14-18].

It has been shown that hypoxia induces the biosynthesis of dopamine[14] and dopamine release[15,27] in type I cells. Moreover, the marked increase of TH—the rate-limiting enzyme in the biosynthesis of catecholamines—in type I cells is induced by hypoxia for 2 weeks[18]. Gene expression for TH is stimulated by hypoxia in type I cells[28,29]. Similar elevations of TH mRNA in the carotid body has been shown *in vitro*[30], while the expression of TH mRNA in the carotid body did not occur in hypercapnia[29] since TH gene expression is regulated by a reduction in oxygen tension. On the other hand, 35 days exposure to hypoxia has been reported to elicit complete disappearance of TH immunoreactivity in chicken glomus cells[31]. An

arousal from hibernation occurs once for a period up to 3-10 days during hibernation season.

In the present study, the immunoreactivity to TH of type I cells increased in hibernating animals, but this increase was reduced during 2 hours after onset of arousal from hibernation. Gauda suggests dopamine and norepinephrine play an inhibitory role in hypoxic chemosensitivity[32], because dopamine receptors may be located on blood vessels and modify blood flow to and within the carotid body, thereby indirectly affecting carotid body chemosensitivity[33]. In this study we found that the diameter of sinusoidal capillaries around type I cells was 13% smaller in hibernating chipmunks than in non-hibernating animals. This may suggest that TH increase during hibernation is involved in the storage of TH, which enables rapid dopamine release during arousal from hibernation and contributes to the carotid body chemosensitivity by controlling blood flow through blood vessel within the carotid body. On the other hand, there has been a report that intra-arterial infusion of catecholamine and L-DOPA causes arousal from hibernation[34]. TH increase during hibernation may be involved in the arousal from hibernation, during which drastic decrease of TH occurs.

Using high-performance liquid chromatography it has been found that the endogenous opioids in the hypothalamus and the septal region increase in the hibernating state[4]. The duration of hibernation was reduced by the continuous infusion of Met-Enk into the lateral ventricle[2]. Close intra-arterial injection of Met-Enk inhibits carotid body chemoreceptors, and this effect is blocked by naloxone, an opiate antagonist[35]. Endogenous opioids depress chemosensitivity during hypoxia[36]. Our present data have shown that the intensity of immunoreactive cells for Met-Enk in type I cells increased during hibernation. It is considered that Met-Enk in type I cells might influence the maintenance of chemoreceptor function during hibernation or arousal from hibernation.

It has been reported that GABA immunoreactivity occurs in almost all type I cells of the mouse carotid body[13,26]. In the present study, numerous GABA immunoreactive type I cells were found in both non-hibernating and hibernating carotid bodies, however, the immunostaining of GABA was stronger in hibernating animals than in non-hibernating animals. GABA is known as the principal inhibitory neurotransmitter in the central nervous system, and GABA is significantly elevated in the cortex and cerebellum of hamsters during hibernation[1]. On the other hand, it has been shown that the extracellular concentration of GABA in the striatum of arctic ground squirrels decreases by 50% during hibernation[5]. It therefore remains unclear whether the GABA increase in type I cells participates in chemoreception during hibernation.

In the present study we found a significant extension of the longitudinal axis of type I cells in hibernating animals ($p<0.001$). Previous investigations under hypoxic condition demonstrated that chronic hypoxia induces a several fold increase of the diameter of both the rat carotid body[16] and the human carotid body[37]. However, in hibernating chipmunks we have observed an increase of the longitudinal axis of type I cells of 22.5%.

The mean diameter of sinusoidal capillaries of the carotid body in the size range of 3-10 μm was 13% less in hibernating animals compared with the control animals ($p<0.001$), which was highlighted with the previous finding that the blood vessels in the carotid body were enlarged by the hypoxia[16,17]. The vascular enlargement by chronic hypoxia increases blood flow in the carotid body and, in contrast, the vascular reduction of the carotid body during hibernation causes the decrease of blood flow via drastic physiological changes. These results would indicate that there are some morphological differences in the carotid body under the hibernating condition and the hypoxic one.

Although hibernation is considered to be a severe hypoxic condition, there are several differences between hypoxia and hibernation. Hibernation is associated with a drastic decrease in oxygen consumption and a reduction in energy metabolism and cellular function. Moreover hibernation is as-

sociated with tolerance to hypothermia and to deprivation of oxygen and glucose[38].

The arterial blood during hibernation was florid, indicating higher arterial oxygen saturation because at low temperature during hibernation the hemoglobin had stored a large amount of oxygen accompanied with the hypothermia. However, immediately after arousal began, a rapid change in arterial blood color from florid to red-brown was observed. These observations suggest that oxygen stored in the hemoglobin during hibernation is depleted immediately, presumably because of the high oxygen consumption during arousal from hibernation due to physiological actions. It is considered that the hypoxic condition dose not occur during hibernation, but occurs during the course of arousal from hibernation.

Periodic arousals during hibernation result in a rapid oxygen uptake and require the accurate regulation of respiratory rate. Therefore it seems that the role of carotid body chemoreceptors is more important during hibernation. It may be that the increases in neuroactive substances—such as TH, Met-Enk and GABA—and the enlargement of type I cells during hibernation result from the demands of drastic respiration changes during periodic arousals. The present data on the increase of immunoreactivity to TH, Met-Enk, and GABA during hibernation suggest that several neuroactive substances in type I cells play a significant role in the regulation mechanism of chemoreception which occurs in response to the hibernating physiological state.

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