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ORIGINAL PAPER

Induction of hibernation-like hypothermia by central activation of the A1 adenosine receptor in a non-hibernator, the rat

Hiroki Shimaoka[1] · Takayuki Kawaguchi[2] · Kahori Morikawa[2] · Yuuki Sano[1] · Kiyotada Naitou[1] · Hiroyuki Nakamori[1] · Takahiko Shiina[1,2] · Yasutake Shimizu[1,2,3]

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Abstract Central adenosine A1-receptor (A1AR)-mediated signals play a role in the induction of hibernation. We determined whether activation of the central A1AR enables rats to maintain normal sinus rhythm even after their body temperature has decreased to less than 20 °C. Intracerebroventricular injection of an adenosine A1 agonist, N6-cyclohexyladenosine (CHA), followed by cooling decreased the body temperature of rats to less than 20 °C. Normal sinus rhythm was fundamentally maintained during the extreme hypothermia. In contrast, forced induction of hypothermia by cooling anesthetized rats caused cardiac arrest. Additional administration of pentobarbital to rats in which hypothermia was induced by CHA also caused cardiac arrest, suggesting that the operation of some beneficial mechanisms that are not activated under anesthesia may be essential to keep heart beat under the hypothermia. These results suggest that central A1AR-mediated signals in the absence of anesthetics would provide an appropriate condition for maintaining normal sinus rhythm during extreme hypothermia.

✉ Yasutake Shimizu
yshimizu@gifu-u.ac.jp

[1] Department of Basic Veterinary Science, Laboratory of Physiology, The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

[2] Laboratory of Veterinary Physiology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

[3] Center for Highly Advanced Integration of Nano and Life Sciences (G-CHAIN), Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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Introduction

Body temperature of hibernators, including hamsters and ground squirrels, drops to only a few degrees above ambient temperature [1, 2]. Although heart rate in hibernating animals is dramatically lowered compared with that in euthermic counterparts, normal sinus rhythm is maintained [3–5]. In contrast, non-hibernating mammals develop ventricular dysfunction and arrhythmias such as atrioventricular block and ventricular fibrillation when their body temperature drops to less than 20 °C [6, 7]. Since extreme hypothermia results in reduction of cellular metabolic rates and oxygen consumption, it may have benefits for the treatment of reperfusion injury in ischemic diseases. Thus, induction of hypothermia in non-hibernating mammals including humans is a challenging endeavor.

It has been demonstrated that central adenosine A1-receptor (A1AR)-mediated signals play a role in the induction and maintenance of hibernation [8–10]. In accordance with this, we previously demonstrated that central administration of an A1AR agonist and subsequent cooling induce extreme hypothermia in hamsters [11]. The predominant role of A1AR-mediated signals leads to the idea that A1AR would induce hypothermia also in non-hibernating mammals. During our trial to test this idea, an important paper was published by Tupone et al. [12]. They clearly showed that central administration of an A1AR agonist in rats exposed to a cool (15 °C) ambient temperature causes a fall in body temperature to about 25 °C due to inhibition of brown adipose tissue and shivering thermogenesis. Accordingly, the potential for central activation

of A1AR to induce a hypothermic state in non-hibernating mammals was proven.

In hamsters, forced hypothermia being comparable to that in hibernating animals can be successfully induced by pentobarbital anesthesia combined with cooling [11]. In this condition, however, notable atrioventricular block was observed. Furthermore, J waves, which are typically observed during hypothermia in non-hibernators [13, 14], were recorded on an electrocardiogram (ECG). On the other hand, central activation of A1AR induced hypothermia without accompanying atrioventricular block or abnormal ECG [11]. These findings raise the possibility that central A1AR-mediated signals may provide an appropriate condition for maintaining normal cardiac pulsatility in addition to their ability to induce hypothermia. To test this possibility, body temperature of rats needs to be dropped to a very low temperature. Thus, the aim of the present study was to examine whether normal sinus rhythm is maintained if body temperature of rats administered an A1AR agonist is forcibly dropped to less than 20 °C.

Materials and methods

Animals

Male Sprague–Dawley rats ($n = 21$, 300–450 g, 8–16 weeks, Japan SLC, Inc., Shizuoka, Japan) were used. The rats were maintained in plastic cages at 22 °C with a 12:12 h light:dark cycle (light on 07:00–19:00 h) and they were supplied with both laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum prior to experiments.

Intracerebroventricular administration of N6-cyclohexyladenosine (CHA)

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (SR-6N; Narishige Scientific Laboratory, Tokyo, Japan). The skull of each rat was exposed and the bregma was located, and then a stainless-steel guide cannula (AG-9; Eicom, Kyoto, Japan) was unilaterally implanted into the lateral ventricle (−4.0 mm AP, 5.0 mm L, 7.0 mm DV), according to the rat brain atlas [15]. The guide cannula was fixed to the calvaria with miniature stainless screws and acrylic dental cement and was plugged with a solid dummy cannula (AD-9; Eicom). Rats were allowed at least one week for recovery. N6-cyclohexyladenosine (CHA) (Sigma, St. Louis, MO, USA) was dissolved in an artificial cerebral fluid (aCSF): NaCl 125, KCl 2.5, $MgCl_2 \cdot 6H_2O$ 1.18, Na_2HPO_4 2.0 (in mM), adjusted to pH 7.4. Twenty nmol of CHA in 5 µl of aCSF was administered through a microinjection cannula (AMI-9; Eicom).

Induction of hypothermia

The conscious rats were administrated with CHA (20 nmol/head, i.c.v.) in an ambient temperature of 22 °C. After administration, the rats were transferred to an ambient temperature of 4 °C to induce extreme hypothermia. We considered that hypothermia was established when the body temperature of the rats reached 15 °C. In another series of experiments, hypothermia was induced forcibly by the combination of anesthesia and cooling. After intraperitoneal injection of sodium pentobarbital (50 mg/kg), the rats were immediately transferred to a refrigerator kept at 4 °C.

If it was necessary to allow recovery of body temperature, hypothermic rats were warmed by using a heating blanket (Homeothermic blanket system, Harvard Apparatus, Holliston, MA, USA).

Measurement of body temperature

In rats injected with CHA, body temperature was monitored using a telemetry system (DAS-5002; BioMedic Data Systems Inc, Seaford, DE, USA), the transmitter (IPTT-200; BioMedic Data Systems Inc, Seaford, DE, USA) of which was placed subcutaneously, until hypothermia-induced immobility was established below 25 °C. Then a small thermistor was inserted 5 cm into the rectum. When anesthetics were used for induction of hypothermia, the thermistor was set at the beginning of cooling.

ECG recording

An ECG was recorded from cable lead electrodes (ECG safety cable lead sets; Philips Medical Systems, USA) placed at the forelimbs with a ground electrode placed at one hindlimb. In rats injected with CHA, the electrodes were set when hypothermia-induced immobility was established (i.e., the same timing as that of thermistor insertion into the rectum). The signal was amplified and displayed on a recorder (M1117A; Hewlett Packard, Palo Alto, CA, USA).

Results

Induction of hypothermia by intracerebroventricular injection of an adenosine A1-receptor agonist in rats

Intracerebroventricular injection of an A1AR agonist, CHA, brought about torpor within 1 h after injection. During the initial torpid period, body temperature was decreased slightly. After confirming torpor, rats were transferred from a warm room kept at 22 °C to a refrigerator kept at 4 °C. Under the cold environment, body temperature of the rats was decreased in a time-dependent manner (Fig. 1). Placement of the rats at 4 °C for a long time decreased body temperature to around 10 °C, resulting in cardiac arrest (data not shown). To avoid cardiac arrest, we transferred rats to a warm room kept at 22 °C when body temperature had dropped to 15 °C. In contrast, rats transferred to a refrigerator immediately after CHA injection (i.e., before establishment of torpor) showed shivering and their body temperature did not decrease.

When rats become immobile at around 25 °C, their heart rate was 128 ± 50 beats/min. Heart rate gradually declined as body temperature decreased. The heart rate of rats for which body temperature had reached 15 °C was 47 ± 9 beats/min. As shown in Fig. 2a, b, regular sinus rhythm was fundamentally maintained at any time point during reduction of body temperature. Skipped heartbeats were occasionally observed (Fig. 2c).

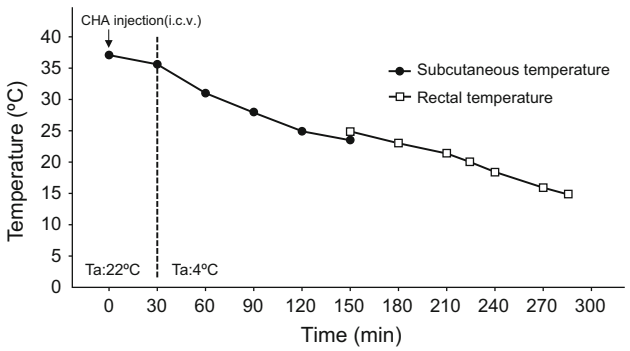

Fig. 1 Central A1AR activation combined with cooling induced hypothermia in rats. A typical graph showing changes in body temperature of the rat injected with CHA (20 nmol/head, i.c.v.). The rat was transferred to a cold room kept ambient temperature (T_a) at 4 °C after confirming torpor. *Filled circles* and *open squares* show subcutaneous and rectal temperatures, respectively. Rectal temperature was measured by inserting a thermistor into the anus after the rat had been immobilized by hypothermia. Body temperature sequentially decreased and finally reached 15 °C. Similar results were obtained in nine independent experiments (the time required for body temperature to reach 15 °C being 312 ± 88 min)

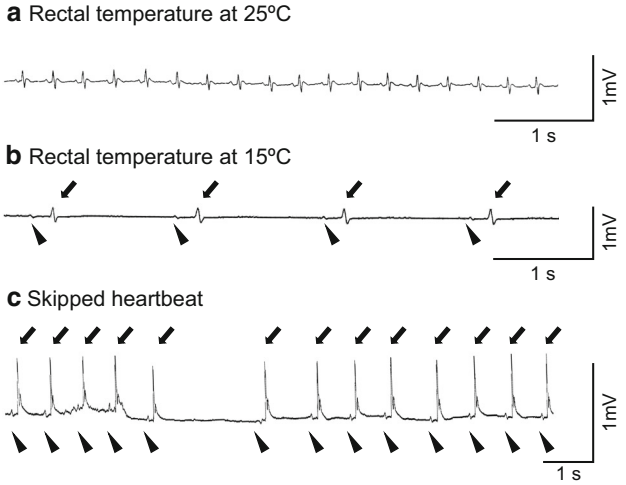

Fig. 2 ECG recorded from a rat in which hypothermia was induced by CHA and cooling. Representative ECG tracings recorded from a rat in which hypothermia was induced by CHA and cooling are shown. P wave and QRS complex are indicated by *arrowheads* and *arrows*, respectively. The rat maintained regular sinus rhythm during hypothermia (**a** at 25 °C and **b** at 15 °C). Skipped heartbeats were occasionally observed (**c**). Similar results were obtained in nine independent experiments

Recovery from CHA-induced hypothermia

To determine whether the induction of hypothermia by injection of CHA is harmful for rats, rats for which body temperature had reached 15 °C were allowed to recover by transferring them to a warm room (22 °C) and warming by using a heating blanket. Heart rate increased as body temperature increased. In the process of recovery, the rats fundamentally maintained normal sinus rhythm and skipped heartbeats were occasionally observed (data not shown). Although rats remained immobile during the recovery phase, spontaneous locomotion and shivering were observed when their body temperature became more than 30 °C.

Induction of artificial hypothermia by cooling in anesthetized rats

For comparison to a condition without central A1AR activation, we induced hypothermia forcibly by the combination of anesthetic injection and cooling. Body temperature was decreased chronologically by transferring rats anesthetized with sodium pentobarbital to a refrigerator kept at 4 °C (Fig. 3a). Heartbeat was maintained in all rats until their body temperature dropped to 25 °C, though heart rate decreased as body temperature decreased (Fig. 3b). At the temperature of 25 °C, an ECG did not show abnormal

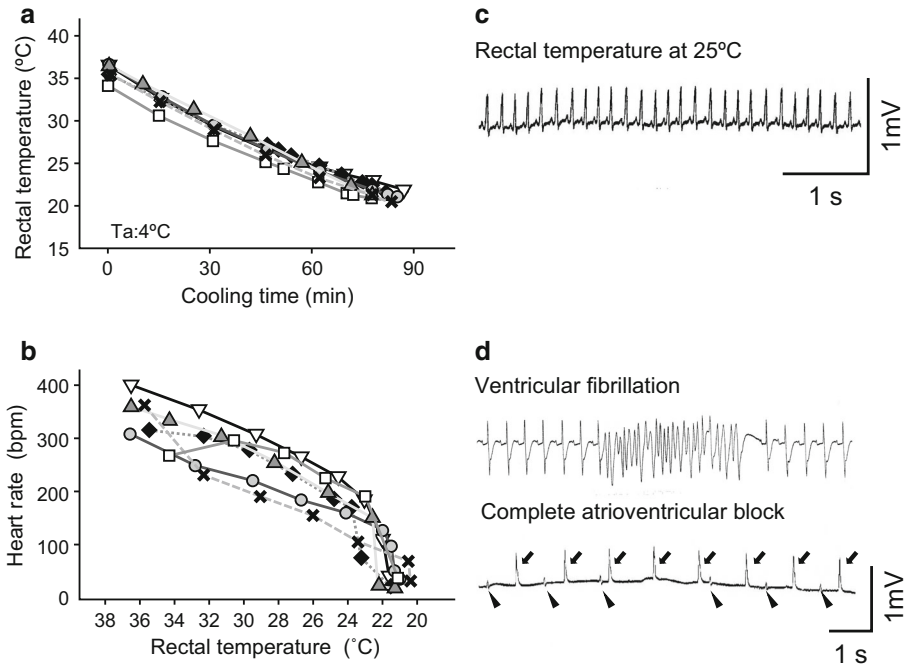

Fig. 3 Abnormal ECG recorded during induction of hypothermia in rats anesthetized with sodium pentobarbital. Rectal temperature of rats injected with sodium pentobarbital (50 mg/kg, i.p.) decreased chronologically in each experiment ($n = 6$) (**a**). Heart rate decreased constantly until body temperature dropped to 25 °C. When body temperature dropped below 22.5 °C, heart rate rapidly decreased (**b**). Abnormal ECGs were not recorded when rectal temperature was above 25 °C (**c**). In contrast, when rectal temperature dropped to about 22.5 °C, complete atrioventricular block and ventricular fibrillation were observed in three and one of the six rats, respectively (**d**). P wave and QRS complex are indicated by *arrowheads* and *arrows*, respectively

waves (Fig. 3c). When the body temperature dropped below 22.5 °C, heart rate rapidly decreased (Fig. 3b). Complete atrioventricular block and ventricular fibrillation were observed in three and in one of the six rats, respectively (Fig. 3d). Regardless of the presence of abnormal ECGs, all of the rats showed cardiac arrest. These results are in contrast to those for a hibernator, the hamster, in which heartbeat is maintained in the same hypothermic condition [11].

Effects of pentobarbital injection on CHA-induced hypothermia

We hypothesized that normal sinus rhythm in CHA-induced hypothermia is due to the operation of some beneficial mechanisms that are not activated in the condition of anesthesia. To test this hypothesis, rats in which hypothermia was induced by CHA were additionally given pentobarbital by intraperitoneal injection. An abnormal ECG reflecting the occurrence of ventricular fibrillation was observed for two of the six rats, and complete atrioventricular block was clearly indicated for three of the six rats (Fig. 4). Although one rat did not show an abnormal ECG, injection of pentobarbital resulted in cardiac arrest in all rats.

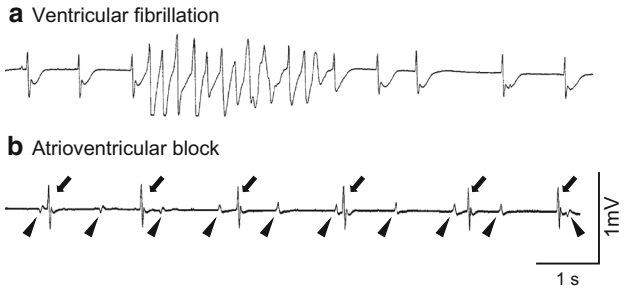

Fig. 4 Effects of pentobarbital injection on CHA-induced hypothermia. Rats in which hypothermia was induced by the combination of CHA injection and cooling were additionally given pentobarbital by intraperitoneal injection and ECG was recorded. Representative abnormal ECGs recorded from these rats are shown. ECGs reflecting the occurrence of ventricular fibrillation (**a**) and complete atrioventricular block (**b**) were observed in two and three of the six rats, respectively. P wave and QRS complex are indicated by *arrowheads* and *arrows*, respectively

Discussion

It has been demonstrated that central administration of an A1AR agonist in rats exposed to a cool (15 °C) ambient temperature causes a fall in body temperature to about 25 °C due to inhibition of brown adipose tissue and shivering thermogenesis [12]. Here, we expanded that work and showed that central administration of an A1AR agonist can

induce extreme hypothermia of less than 20 °C in rats, even though rats are not hibernators. The main findings of this study are: (1) transferring rats to a refrigerator kept at 4 °C after intracerebroventricular injection of an A1AR agonist, CHA, successfully decreased their body temperature to less than 20 °C, (2) regular sinus rhythm was fundamentally maintained at any time point of hypothermia, though skipped heartbeats were occasionally observed, and (3) rats in which hypothermia was induced by injection of CHA and cooling can recover by being transferred to a warm room kept at 22 °C and warming. Considering that hypothermia forcibly induced by the combination of anesthetic injection and cooling in rats brought about cardiac arrest, our results indicate that central activation of A1AR would provide an appropriate condition for maintaining heartbeat during extreme hypothermia.

Intracerebroventricular injection of CHA induced torpor in rats even though they were kept at room temperature. The fact that body temperature decreased during the initial torpid period suggests that CHA suppresses the central mechanism responsible for regulating body temperature. Of note, rats transferred to a refrigerator kept at 4 °C immediately after injection of CHA showed shivering and did not enter a deep hypothermic state. The result seems to conflict with the reported observation by Tupone et al. [12]. They observed that intracerebroventricular injection of CHA to rats maintained in an ambient temperature of 15 °C caused a decline in body temperature. This discrepancy is probably due to the difference in the ambient temperature to which rats were exposed after the injection of CHA; that is, we exposed the rats to ambient temperature of 4 °C by transferring them from a temperature of 22 °C. The A1AR-mediated hypothermia would be due to inhibition of thermogenesis in brown adipose tissue caused by activation of the sympathoinhibitory pathway emanating from the intermediate nucleus of the solitary tract [12]. Since a cold ambient temperature is the most suitable stimulus for activating brown adipose tissue thermogenesis, exposure to a severe cold temperature may drive competing signals for the A1AR-mediated inhibitory pathway. The activating pathway triggered by a cold temperature can exceed the A1AR-mediated inhibitory pathway during the initial stage of the inhibitory pathway operation. Our results also suggest that the activation signals for thermogenesis cannot reverse the A1AR-mediated inhibitory pathway when the inhibitory pathway have well operated.

Unlike hibernators including hamsters, non-hibernators cannot maintain their heartbeat under a hypothermic condition of less than 20 °C [6, 7]. In fact, we found that induction of extreme hypothermia by the combination of anesthetic injection and cooling brought about cardiac arrest in rats. The result is in contrast to that for a hibernator, the hamster, in which heartbeat is maintained in the same hypothermic condition [11]. These facts clearly demonstrate that the hearts of hibernators are highly tolerant to cold temperatures compared with the hearts of non-hibernators. However, the cardiac arrest observed after inducing extreme hypothermia in rats seems unlikely to be totally associated with innate properties of the heart, because heartbeat was maintained in rats in which body temperature decreased to less than 20 °C after central administration of CHA. More importantly, the heart under an extreme hypothermic condition would suffer, if any, little damage, as judged by the fact that rats could recover with warming after body temperature had reached 15 °C. Accordingly, it is reasonable to assume that some beneficial mechanisms operate during CHA-induced hypothermia.

Our results suggest that the putative beneficial mechanisms do not operate under anesthesia. Considering that autonomic nervous activities are generally suppressed in animals treated with anesthesia, it is possible that the mechanisms are related at least in part to autonomic controls. Several lines of evidence have indicated that autonomic controls are still present and are involved in heartbeat regulation during natural hibernation [3, 16–18]. Imbalance of the autonomic nervous system can result in cardiac arrhythmias and myocardial damage. For instance, a neurogenic stunned myocardium, which is defined as transient left ventricular dysfunction occurring after acute brain injury, has been suggested to be due to an imbalance of the autonomic nervous system [19]. Thus, proper autonomic controls may be necessary for the maintenance of normal sinus rhythm under an extreme hypothermic condition.

In summary, the present study showed that it is possible to induce hibernation-like extreme hypothermia in a non-hibernator, the rat. Central activation of A1AR is an effective method for inducing hypothermia of less than 20 °C in the absence of apparent problems in the heart. Since administration of anesthetics to the hypothermic animals brings about cardiac arrest, it is probable that the operation of some beneficial mechanisms that are not activated in the condition of anesthesia is essential to keep heart beat under the hypothermic condition.

Author contributions HS, TK and KM contributed to collection, analysis and interpretation of data and wrote the article. YSa, KN and HN performed acquisition of data. TS participated in the design of the study and helped to draft the manuscript. YSh was a supervisor in this study and revised the article critically for important intellectual content. All authors read and approved the final manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The experimental procedures were performed according to the guidelines for the care and use of laboratory animals approved by the Animal Care and Use Committee of Gifu University (permission numbers: 14102 and 15096). This article does not contain any studies with human participants performed by any of the authors.

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