Type 2 diabetic mice enter a state of spontaneous hibernation-like suspended animation following accumulation of uric acid

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Hibernation is an example of extreme hypometabolic behavior. How mammals achieve such a state of suspended animation remains unclear. Here we show that several strains of type 2 diabetic mice spontaneously enter into hibernation-like suspended animation (HLSA) in cold temperatures. Nondiabetic mice injected with ATP mimic the severe hypothermia analogous to that observed in diabetic mice. We identified that uric acid, an ATP metabolite, is a key molecular in the entry of HLSA. Uric acid binds to the Na⁺ binding pocket of the Na⁺/H⁺ exchanger protein and inhibits its activity, acidifying the cytoplasm and triggering a drop in metabolic rate. The suppression of uric acid biosynthesis blocks the occurrence of HLSA, and hyperuricemic mice induced by treatment with an uricase inhibitor can spontaneously enter into HLSA similar to that observed in type 2 diabetic mice. In rats and dogs, injection of ATP induces a reversible state of HLSA similar to that seen in mice. However, ATP injection fails to induce HLSA in pigs due to the lack of their ability to accumulate uric acid. Our results raise the possibility that nonhibernating mammals could spontaneously undergo HLSA upon accumulation of ATP metabolite, uric acid.

Torpor and hibernation are traditionally defined as two different types of hypometabolic states under natural conditions (10). Different from torpor, animals under hibernation display much lower body temperatures and metabolic rates (11). A prominent physiologic and behavioral characteristic of hibernation is suspended animation, associated with tolerance to lethal metabolic rate reduction, bradycardia, and profound hypothermia (12). Some chemical compounds have been used to induce a hibernation-like suspended animation (HLSA) in nonhibernating animals. Hydrogen sulfide (H₂S) experimentally induces suspended animation in mice, and they will return to normal temperature after H₂S removal (13). However, H₂S has been reported to be toxic to the livers in both animals and humans (14). Another chemical, AMP, induces a reversible deep hypometabolic state in nonhibernating mice, which is assumed to be caused by increased blood 2,3-bisphosphoglycerate and inhibited binding of oxygen to red blood cells (15). In nature, the decline in body temperature is closely related to the adenosine signal of A₁ receptor in the brain (16). The central inhibition of A₁ receptor in the brain reverses the temperature rise of ground squirrels during hibernation, indicating that adenosine could play an essential role in adjusting body temperature (16).

The physiological definition of hibernation is not yet clear. During natural hibernation, glucose metabolism via glycolysis seems to be strongly suppressed. Inhibition of glycolysis readily induces hypothermia in a classic hibernator, indicating that the underlying mechanism of hibernation could be metabolic suppression (17). Indeed, the ability to replicate this naturally hibernating status has been achieved using metabolic inhibitors alone in rodents such as mice and rats (18). Insulin resistance is significant in the fattening period of hibernating animals. During the prehibernation period, serum insulin levels are elevated and maintain a high level in the initial months of hibernation (19). The actions of chronically high insulin levels are usually associated with insulin resistance in type 2 diabetes (T2DM). Lack of insulin response has also been reported in hibernating dormice and hedgehogs (20, 21). These observations imply there may be some similar metabolic

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regulation mechanisms between type 2 diabetic mice and hibernating animals.

Here, we found that several strains of type 2 diabetic mice spontaneously enter into a hibernation-like suspended animation in cold temperatures. Nondiabetic mice with ATP injection mimic the severe hypothermia state similar to that observed in the type 2 diabetic mice. We identified that the accumulation of uric acid from ATP metabolism is an indispensable step in the induction of HLSA. Uric acid is an inhibitor of \( \text{Na}^+ / \text{H}^+ \) exchanger, which controls the pH homeostasis of cytoplasm and influences the activities of a series of metabolic enzymes. It is well known that uric acid accumulation is a common feature of animals in long-term food shortage or fasting (22–25), and natural hibernation always commences when food is absent or limited (10). Our results strongly suggest that uric-acid-regulated metabolic suppression reflects not only a drop of metabolic activity during the development of T2DM, but also associates the metabolic regulation mechanism of natural hibernation.

**Results**

**HLSA of the type 2 diabetic mice**

Severe hypothermia observed during hibernation is a form of suspended animation (26). Accumulated evidence indicates that metabolic suppression must be a key factor to achieve hibernation (26). Hypometabolic behavior is also the main feature of T2DM. Therefore, we undertook a study to determine whether there were differential responses in body temperature (T\(_b\)) and locomotive activity while type 2 diabetic mice with severe metabolic stress were exposed to cold temperatures. Dramatically, the \( \text{db/db} \) diabetic mice displayed none of the thermoregulatory defenses when maintained in the environment with ambient temperatures (T\(_a\)) around the freezing point (0 °C). T\(_b\) measurement revealed a rapid decline accompanied by distinct behavioral responses with physical inactivity (Fig. 1A, left). About 1–3 h (stage I) after cold exposure, T\(_b\) of mice dropped to 18 ± 0.5 °C, and mice entered HLSA. In this stage, mice lost the righting reflex when placed on their backs or sides. The HLSA in type 2 diabetic \( \text{db/db} \) mice would last for several hours by adjusting the T\(_a\) of 17 ± 0.5 °C (stage II). Then, mice would arouse from HLSA spontaneously or be awakened when T\(_a\) was adjusted to more than 32 °C. This stage would last for about 2–3 h until the T\(_b\) of mice was close to 36 to 37 °C, and the moving activity was recovered (stage III). Simultaneously, the T\(_b\) in control mice was within the normal range. These differences were confirmed by surface thermal imaging (Fig. 1A, right).

The same HLSA has also been observed in HFD-STZ diabetic mice, while chow-fed lean control mice maintained a relatively constant T\(_b\) (Fig. 1B). Similarly, diabetic \( \text{ob/ob} \) mice entered into HLSA in cold temperatures, while nondiabetic \( \text{ob/ob} \) mice tended to reduce T\(_b\) and failed to enter into HLSA (Fig. 1C). During the HLSA in diabetic \( \text{ob/ob} \) mice, the average heart rate and respiratory rate also declined to about 69 beats per minute and 18 times per minute, respectively (Table 1). Moreover, all groups of type 2 diabetic mice showed a severe decrease in spontaneous locomotive activity compared with control mice (Fig. 1, D–F). While oxygen consumption was relatively stable in control mice in cold temperatures, all type 2 diabetic mice decreased oxygen consumption accompanied by the drop in T\(_b\) (Fig. 1, G–I). These observations indicated that laboratory type 2 diabetic mouse models are capable of entering a suspended animation state similar to that observed in hibernators.

**ATP induces HLSA in mice**

The above observations could be explained by a changed metabolite that acted as a metabolic repressor in type 2 diabetic mice. We reasoned that the putative metabolic repressor, when injected into wild-type mice under cold exposure, should induce HLSA as observed in diabetic mice. Then we investigated whether endogenous energy molecules in diabetic mice were different from those in nondiabetic mice. HPLC analysis revealed that ATP metabolites, including hypoxanthine, xanthine, and uric acid, were elevated in the plasma and livers of diabetic \( \text{ob/ob} \) mice compared with nondiabetic \( \text{ob/ob} \) mice (Fig. 2, A–D). We hypothesized that the changes in endogenous energy molecules must be a key factor to achieve HLSA in diabetic mice.

Therefore, we undertook a study to determine whether there were differential responses in T\(_b\) while administrating endogenous energy molecules in animals. The wild-type mice were given adenosine triphosphate (ATP 2Na salt) and then maintained at 4 °C of T\(_a\). They displayed no thermoregulatory defenses as observed in diabetic mice under cold exposure. T\(_b\) measurement revealed a rapid decline accompanied by distinct physical inactivity (Fig. 2E). About 30 min (stage I) after ATP injection, T\(_b\) dropped to 16 ± 0.5 °C, and mice entered the HLSA (stage II). T\(_b\) drop caused by ATP was clearly detected with an infrared thermometer (Fig. 2F), and a severe decrease of locomotive activity compared with control mice was measured by a radio frequency receiver platform (Fig. 2G). HLSA would last for about 6–8 h. Then, mice would arouse from HLSA spontaneously or be awakened when T\(_a\) was adjusted to 25 °C, and the moving activity was recovered (stage III). The changes in oxygen consumption corresponded with the major decline in T\(_b\) (Fig. 2H). During the HLSA in mice, the average heart rate and respiratory rate also declined to about 85 beats per minute and 13 times per minute, respectively (Table 2). The concentrations of ATP are important for entering HLSA. With the increase of ATP dosage, the entry time of HLSA was shortened (Fig. 2I). Together, these studies showed that ATP is a signaling molecule that can activate HLSA, which is used as a mechanism for energy conservation.

**ATP-induced HLSA is independent of purinergic receptors**

Next, we compared the effects of ATP and its downstream products, AMP and adenosine, on the induction of hypothermia. At a T\(_a\) of about 4 °C, ATP induced 100% of mice to HLSA, while AMP and adenosine were about 80% and 60%, respectively (Fig. 3A). The HLSA entry time of ATP was shorter than that of AMP and adenosine (Fig. 3B). In order to
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Figure 1. Cold exposure induces a hibernation-like suspended animation in diabetic mice but not in nondiabetic mice. A, simultaneous measurements of body temperature ($T_b$) in db/db mice or lean mice after cold treatment. Data were presented as mean ± range (N = 6). Thermal images showing surface temperature difference at 3 h after cold treatment. The dashed line represents the division between stages. Scale bar: 2 cm. Stage I: induction stage; Stage II: maintenance stage; Stage III: awakening stage. B, simultaneous measurements of $T_b$ in diabetic mice or nondiabetic mice after cold treatment. Data were presented as mean ± range (N = 6). Thermal images showing surface temperature difference at 3 h after cold treatment. Scale bar: 2 cm. C, simultaneous measurements of $T_b$ in diabetic mice or nondiabetic db/db mice after cold treatment. Data were presented as mean ± range (N = 6). D–I, O$_2$ consumption between db/db mice and lean mice (D), HFD-STZ mice and chow-fed control mice (E), diabetic ob/ob mice, and nondiabetic ob/ob mice (F). The dashed line indicates cold exposure onset. G–I, O$_2$ consumption between db/db mice and lean mice (G), HFD-STZ mice and chow-fed control mice (H), diabetic ob/ob mice, and nondiabetic ob/ob mice (I). The dashed line indicates cold exposure onset. Data were presented as mean ± SD (N = 6).

determine whether the specific role of ATP was performed by its receptor P$_2$X or P$_2$Y, we used the antagonists of P$_2$X and P$_2$Y. While P$_2$X antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) had no effect on the induction of ATP, the antagonist of P$_2$Y receptor suramin significantly prolonged the induction period of ATP (Fig. 3C). However, no matter how high the dose of suramin is, it could not completely block mice from ATP-induced HLSA.

**Table 1**

| Changes in heart rate and respiratory rate in nondiabetic ob/ob mice and diabetic ob/ob mice after cold treatment |
|----------------------------------------------------------------------------------------------------------------|
| **Group**                              | **Heart rate (bpm)** | **Respiratory rate (bpm)** |
|----------------------------------------|---------------------|---------------------------|
| Nondiabetic ob/ob (N = 6)              | 473 ± 66            | 85 ± 21                   |
| Diabetic ob/ob (N = 6)                 | 69 ± 11*            | 18 ± 7**                  |

Data were presented as mean ± SD (Student’s t test: *p < 0.01).

* Beats per minute.

**ATP metabolites acidify cytoplasm**

Because ATP-induced hypothermia is immediate, we reasoned that ATP action in this process was very fast, and it could not be achieved through complex regulation of gene and protein expression, although some of them may have a rapid
Figure 2. Elevated nucleotide levels in diabetic mice and ATP can induce HLSA in wild-type mice. A and B, quantification of adenine nucleotides (A) and adenine nucleotide metabolites (ANMs) (B), including inosine, hypoxanthine (Hyp), xanthine (Xan), and uric acid (UA), in the plasma of diabetic ob/ob mice compared with nondiabetic ob/ob mice. Data were presented as mean ± SD (N = 6; Student’s t test: *p < 0.05, **p < 0.01). C and D, quantification of adenine nucleotides (C) and ANMs (D), including adenosine (Ado), Hyp, Xan, and UA, in the liver of diabetic ob/ob mice, compared with nondiabetic ob/ob mice. Data were presented as mean ± SD (N = 6; Student’s t test: *p < 0.05, **p < 0.01). E, Simultaneous measurements of Tb of mice given ATP (0.5 mg/g, i.p., indicated by a down arrow, red) or saline (black). Data were presented as mean ± range (N = 6). F, representative real-time photographs and digital infrared thermal images of mice after ATP injection. Scale bar: 2 cm. G and H, activity levels (G) and O2 consumption (H) after injection of ATP or saline. Data were presented as mean ± SD (N = 6). I, the entry time of HLSA induced by different ATP doses. Data were presented as mean ± SD (N = 6; ANOVA: *p < 0.05).
response. Then, we investigated whether exogenous nucleotides immediately changed the metabolic environment, such as intracellular pH. Figure 4A showed representative pseudocolored ratio images of intracellular pH value. The results revealed that ATP metabolites, including adenosine, hypoxanthine, xanthine, and uric acid, acidified cytoplasm with different efficiency, and uric acid is the most effective for cytoplasm acidification. Then, several metabolic enzymes would be impaired or lost their catalytic activity. Glucokinase and phosphofructokinase are two rate-limiting enzymes of glycolysis. The activities of these enzymes decreased with the decline of pH (Fig. 4, B and C). Fluorescence intensity assay revealed that glucose uptake was also significantly impaired with uric acid incubation in cultural cells (Fig. 4, D and E).

Since uric acid is the most effective inducer of intracellular acidification and the elevation in UA level and the decrease in body temperature occurred simultaneously (Fig. S1), we investigated whether the inhibition of uric acid accumulation influenced HLSA in mice. Xanthine oxidase is required to participate in the biosynthesis of uric acid. Surprisingly, when mice were pretreated with the inhibitors of xanthine oxidase, fenbuxostat, or allopurinol, ATP failed to induce mice to HLSA (Fig. 4, F and G), nor could diabetic db/db mice (Fig. S2). These pretreated diabetic mice lost the ability to enter stage II and recovered to normal Tb or died within 1–2 h during stage I. Moreover, mice pretreated with a uricase inhibitor potassium oxonate for 7 days developed hyperuricemia and could spontaneously enter into HLSA under cold exposure (Fig. S3). These studies indicated that uric acid suppresses metabolic rate by acidifying cytoplasm, and uric acid is indispensable during ATP-induced HLSA in mice.

### Uric acid is an endogenous inhibitor of Na+/H+ exchanger

To identify the target of uric acid acidifying cell, the relationship between uric acid and the Na+/H+ exchanger activity was investigated. The Na+/H+ exchanger is a key regulator of cellular pH homeostasis. Interestingly, uric acid inhibited the activity of Na+/H+ exchangers in a dose-dependent manner. (Fig. 5, A–C). Na+/H+ exchanger 1 (NHE1) is one of the most important isoforms of Na+/H+ exchanger and is ubiquitously distributed throughout the plasma membrane of virtually all tissues. We investigated whether uric acid could interact with NHE1 proteins. The structure of the transmembrane segments of NHE1 was predicted according to Phyre2 homology modeling portal. We then conducted the molecular docking by AutoDock4.2 software. According to the molecular docking prediction, uric acid displayed a high binding affinity to the extracellular Na+-binding site of NHE1 protein, and the crucial amino acids involved in the NHE1-uric acid binding are SER161, PHE164, PHE165, and PHE467 (Fig. 5D). Then, knockout of the possible uric acid-binding domain (15 amino acid residues from position 156–170) was implemented by overlap extension PCR (Fig. 5E). A microscale thermophoresis assay verified that uric acid was able to bind to NHE1 (Fig. 5F).

Moreover, in the AML12 cells transfected with specific siRNA targeting NHE1, the initial pH5 value was decreased, and the decrease of pH, triggered by uric acid was dampened (Fig. 5H, upper panel). The sequence-optimized NHE1-WT constructs, but not NHE1-mutant, rescued the effects of intracellular acidification reduced by NHE1-siRNA (Fig. 5H, middle and lower panels). Interestingly, the wild-type mice injected intravenously with zoniporide hydrochloride hydrate, a selective inhibitor of NHE1, were successfully induced into HLSA (Fig. 5G). Together, these studies indicated that the primary role of uric acid is as an endogenous inhibitor of Na+/H+ exchanger.

### Mice undergo HLSA have no systemic inflammation or organ damage

To determine whether the mice under HLSA or recovery from HLSA (R-HLSA) have systemic inflammation or organ damages, we evaluated plasma levels of MMP-1, IL-1β, and CRP by ELISA. There were no statistically significant differences in MMP-1, IL-1β, and CRP levels between the HLSA, R-HLSA, and control groups (Fig. 6A, upper left panel). In order to assess the heart, kidney, and liver functions, we measured the plasma levels of creatine kinase-MB (CK-MB), creatinine, blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in all groups. Using plasma CK-MB as a marker of heart injury, we observed no significant increase in plasma CK-MB in the HLSA and R-HLSA groups compared with the control group (Fig. 6A, upper right panel). The levels of BUN and creatinine in plasma increased slightly in HLSA and dropped to the normal range in R-HLSA (Fig. 6A, lower left panel). It should be noted that the HLSA-mediated elevations in plasma AST and ALT compared with the control mice were small and likely represented no damage to liver tissue (Fig. 6A, lower right panel). Measurements of water and food consumption were performed 3 days before and 3 days after a single ATP-induced HLSA, respectively, and there was virtually no change (Fig. 6B, upper panel).

Then, we utilized metabolomics to investigate the metabolic changes in the circulation and main organs during and after HLSA. The PCA analysis was performed on the metabolic...
Figure 3. Purinergic receptor is not required in HLSA. A, different efficiency of ATP, AMP, and adenosine in inducing mice into the HLSA. Mice were injected with the same dose (1 μmol g BW^{-1}) of ATP, AMP, and adenosine, respectively, and were maintained at 4 °C T_b. Data were presented as mean ± SD from four time-independent experiments (N = 20 for each assay; ANOVA: **p < 0.01). B, simultaneous measurement of T_b of mice given ATP (red), AMP (blue), and adenosine (black) (1 μmol g BW^{-1}, indicated by down arrow) in individual metabolic chambers at T_b ~ 4 °C. C, the time of each stage of the hibernation-like state induced by ATP after using receptor antagonist of P2X (suramin, 75 mmol/kg, i.p., 1 h before ATP-injection) or P2Y (PPADS, 140 mmol/kg, i.p., 1 h before ATP-injection). Data were presented as mean ± SD (N = 10; ANOVA: **p < 0.01). D, the time of each stage of the hibernation-like suspended animation induced by ATP in A1−/−, A2a−/−, A2b−/−, and A3−/− mice. No significant differences in HLSA stages in these mice. Data were presented as mean ± SD (N = 10; ANOVA: p > 0.05). E and F, quantification of adenosine nucleotides (E) and ANMs (F), including inosine, Hyp, Xan, and UA in the plasma of ATP-treated wild-type mice compared with saline-treated control mice at 1 h after the injection. Data were presented as mean ± SD (N = 6; Student’s t test: *p < 0.05; **p < 0.01). G and H, quantification of adenine nucleotides (G) and ANMs (H), including Ado, Hyp, Xan, and UA in the liver of ATP-treated wild-type mice compared with saline-treated control mice. Data were presented as mean ± SD (N = 6; Student’s t test: *p < 0.05; **p < 0.01).
Figure 4. ATP metabolites immediately acidify the cytoplasm and UA is indispensable during ATP-induced HLSA. A, representative pseudo-colored ratio image showed a spatial map of intracellular pH (pHi) (color pH scale to the right). a, the left and middle panels showed the difference of pHi, between cells treated with ATP metabolites (adenosine, hypoxanthine, xanthine, and uric acid) for 0 min (left) and 8 min (right), scale bar: 100 μm. b, the right panel showed a time course of change in pHi, after the addition of ATP metabolites. B and C, effect of the pH on the relative activity of glucokinase (B) and phosphofructokinase (C). Data were presented as mean ± SD (N = 5 wells per treatment; note the error bars are smaller than the markers on the plot). D, representative images of AML12 at 0 μM UA (a), 300 μM UA (b), and 500 μM UA (c) for 30 min. Green fluorescence indicated the localization of glucose uptake by cells. Scale bar: 50 μm. E, glucose uptake in AML12 hepatocytes at 30 min after the addition of 300 μM or 500 μM UA measured by an FACScan laser flow cytometer. Data were presented as mean ± SD (N = 6 wells per treatment; ANOVA: *p < 0.05, **p < 0.01). F and G, Tg changes (F) and activity level changes (G) after injection of ATP and xanthine oxidase inhibitors, febuxostat (5 mg/kg, i.p., 1 h before ATP-injection) or allopurinol (20 mg/kg, i.p., 1 h before ATP-injection). Data were presented as mean ± SD (N = 6). Ado, adenosine; Allo, allopurinol; CTRL, control; Febx, febuxostat; POP, propionate; Hyp, hypoxanthine; UA, uric acid; Xan, xanthine.
Figure 5. UA inhibits NHE1 activity and binds to the Na⁺-binding site of NHE1. A and B, effect of 5 μM (A) or 50 μM (B) UA on the recovery of pH, from acidification induced by 20 mmol/l propionate. Original tracings of typical experiments for pH measurement were shown. C, Na⁺/H⁺ exchanger activity was rapidly reduced by 5 μM UA and 50 μM UA. Data were presented as mean ± SD (N = 6; ANOVA: **p < 0.01). D, alignment of the docking poses and key residues in the NHE1 and UA complexes. E, schematic diagram of key amino acid residues knockout. F, MST time traces of two different concentrations of UA were mixed with NHE1-mcherry protein. G, MST time traces of two different concentrations of UA were mixed with NHE1-mcherry mutant protein. H, pseudo-colored ratio image showed a spatial map of intracellular pH (pHi) (color pH scale to the right). The left and middle panels showed the difference of pHi between cells treated with 500 μM UA for 0 min (left) and 8 min (right). The right panel showed a time course of change in pHi after the addition of UA. Scale bar: 200 μm.
profiles of the control, HLSA, and R-HLSA groups. In the PCA score plots from the plasma, liver, kidney, and brain (Fig. 6B, lower panel), the HLSA group was well separated from the control group, while the R-HLSA group and control group clustered together, revealing that with the recovery of Tb, mice can quickly recover from HLSA-induced metabolic disturbance without apparent damage. The corresponding coefficient-coded loading plots (Figs. S5–S8) also elucidated that mice could quickly recover from HLSA-induced metabolic disturbance to their normal status.

To observe the influence of the ATP-induced HLSA on the immune system, flow cytometric analysis of the draining lymph nodes and spleens was performed after mice underwent HLSA once a day for 10 consecutive days. There was no change in the percentages of immature T cells in the lymph nodes (Fig. 6C) and the spleens (Fig. 6D). Together, these results demonstrated that ATP-induced HLSA has no systemic inflammation or organ damage.

ATP induces HLSA in rats and dogs

Next, we investigated the effects of ATP in other mammals. Rats were injected with ATP and then maintained at a Ta of about 4 °C. The behavior response of rats during HLSA was similar to that observed in mice. However, the time of entering HLSA is about 2.5–3 h (stage I) in rats, compared with 0.5 h in mice (Fig. 7A). The Tb of rats in HLSA was close to 16 °C, as same as that of mice. Then, we transferred these rats to a Ta of

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Figure 7. ATP induces HLSA in mice, rats, and beagles, but not in pigs. A, representative curve of $T_a$ changes in mice after ATP injection (1 μmol/g BW). The dashed line represents the division between stages. Stage I: induction stage; Stage II: maintenance stage; Stage III: awakening stage. B, representative curve of $T_a$ changes in rats after ATP injection (1 μmol/g BW). Rats were shaved off prior to ATP injection. Rats can awaken spontaneously undergoing stage II for 6–8 h. C, $T_a$ changes in beagles after ATP injection (0.5 μmol/g BW). Beagles were shaved off prior to ATP injection. During stage II, the beagles were unable to awaken spontaneously until they were transferred to room temperature. D, $T_a$ changes in pigs after ATP injection (0.5 μmol/g BW). Pigs only declined their $T_a$ to 32–33 °C and remained active for several hours. The dashed line indicates cold exposure onset. Down arrow indicates injecting time points, and up arrow indicates transferring time points. E, schematic representation of the proposed regulatory function of ATP on the induction of an HLSA. The model shows ATP causes UA accumulation, thereby acidifying the cytoplasm and inhibiting the production of heat from glucose oxidation phosphorylation.

about 15 °C. The rats would keep HLSA for 6–8 h (stage II). The rats were then awakened by being moved to a $T_a$ of 25 °C. After 3–4 h (stage III), the $T_a$ of the rats would recover to close to 37 °C (Fig. 7B). The time of stage III in mice is about 2–3 h. The rats in stage II could also awaken by elevating $T_a$ at any required time. For dogs, they must be shaved off prior to ATP injection. Shaved dogs were injected with ATP and maintained at a $T_a$ of about 4 °C (Fig. 7C). After 1.5–2 h (stage I), the dogs would enter the HLSA similar to that observed in rats and mice. The $T_a$ of dogs in HLSA was about 18–20 °C. Then, we transferred the dogs to a $T_a$ of 10 °C. The dogs could maintain HLSA for 8–10 h (stage II). The dogs during stage II could not spontaneously arouse at a $T_a$ of 10 °C. However, while the dogs were transferred to a $T_a$ of 25–30 °C, their $T_a$ would recover to about 37 °C within 8–10 h (stage III, Table S1). Especially, when injected with ATP (0.5 μmol/g BW) intraperitoneally at 4 °C, the pigs only reduced their $T_a$ to 32–33 °C and remained active for several hours (Fig. 7D). Pigs injected with ATP (1 μmol/g BW) at 4 °C would die within 8–12 h. ATP could not induce pig to HLSA.

Discussion

Type 2 diabetes and hibernation are two different pathological and physiological phenomena. However, both of them have a common metabolic characteristic that glucose utilization is seriously inhibited. Hibernators are considered a remarkable model for reversible insulin resistance (27). Many hibernators naturally undergo a massive increase in body fat storage before the hibernation season and, to a large extent, rely on triglycerides in white adipose tissue as an energy source in winter (28, 29). For fat-storing hibernators, reduced metabolic rate and body temperature are also accompanied by shifts of fuel utilization and energy-generating pathways from the utilization of carbohydrates to the utilization of fat (26, 30, 31). In T2DM, the actions of chronically high insulin levels are
usually associated with insulin resistance, and metabolic reprogramming results in alterations in fuel utilization (32, 33). The uncoupling of glycolysis from glucose oxidation and increased fatty acid oxidation are considered specific metabolic changes of T2DM. Thus, there is a reasonable physiological and metabolic basis for type 2 diabetic animals to enter a spontaneous HLSA in cold temperatures.

Persistently elevated blood nucleotide levels and constant purinergic signaling may play a pathophysiological role in the development of T2DM (34). Analysis of the relationship between circulating nucleotide concentrations and clinical measures of T2DM demonstrate that changes in nucleotide metabolism are direct metabolic consequences of the disease and do not result from secondary complications (34). High nucleotide levels observed in some morbidly obese subjects seem to be associated with the initial stage of insulin resistance (35). Uric acid is the ultimate product of purine metabolism in the human body. A close association between serum uric acid and abnormal glycometabolism has been well built (36, 37). The current analyses suggest that elevated serum uric acid concentrations have been positively associated with the development of T2DM (37, 38). In our present study, uric acid is an inhibitor of Na+/H+ exchanger, which directly regulates cell pH homeostasis. Acidifying cell suppresses the metabolic rate through influencing a series of metabolic enzymes. The suppression of uric acid biosynthesis blocks the occurrence of HLSA in both type 2 diabetic mice and ATP-treated wild-type mice, suggesting a crucial role of uric acid in the induction of HLSA. On the other hand, ATP interaction with P2 receptors to acidify cellular plasma also plays a partial role in the induction of HLSA. Thus, the entry time of ATP in inducing hibernation is shorter than that of AMP and adenosine. A previous investigation shows that pyruvate, an energy-rich metabolic intermediate, induces hypothermia in diet-induced obese mice (39), which relies on adenosine receptors and GABA signaling. Nevertheless, in our present ATP-induced HLSA, it seems adenosine receptor signaling is not a key trigger. Uric acid accumulation did not influence GABA levels in the cerebral hemisphere, cerebellum, and the medulla oblongata (40, 41). Several regions in the mammalian brain have been implicated in coordinating temperature regulation for the entrance into a hibernation-like state (42–44). While UA generally displays inhibitory effects of metabolism in peripheral tissues, it will be interesting to see whether it affects the thermoregulatory center in the brain.

The fact that ATP fails to induce pig to HLSA further supports our conclusion that higher levels of uric acid accumulation are essential in metabolic suppression. In pigs, uric acid is not a terminal product of ATP, and xanthine oxidase is required to participate in the production of uric acid, which is then metabolized to allantoin by uricase (45). The level of xanthine oxidase in pigs is very low, but the activity of uricase is quite high (46–49). Therefore, it is difficult to accumulate uric acid in pigs. Unlike uric acid, allantoin could not acidify cytoplasm (Fig. S9). In mice and rats, the levels of xanthine oxidase are thousands of times higher than that in pigs, and the levels of uricase are similar to that in pigs (46, 47, 50–52).

Thus, uric acid accumulates sharply following ATP injection in these animals. For humans and primates, uricase is absent in the evolutionary process. Uric acid is a final product from purine derivatives in humans and primates (53), indicating a possibility that ATP induces these large mammals into HLSA.

In natural hibernation, food shortage and reduced ambient temperature are two essential factors for entering an energy-conserving torpid state. For animals that have been deprived of food for a long time, their serum uric acid levels will increase (22). Plasma uric acid levels increased in fasting emperor penguins, accompanied by a lower level of locomotor activity (23). Spontaneously fasting birds increased their serum uric acid levels during migratory flight (24). It was reported that plasma levels of uric acid were increased in human undergoing 10 days of fasting (25), and increased urate in plasma has also been reported in hibernating bears and snakes (54, 55). Therefore, our results strongly suggest that uric-acid-modulated metabolic suppression may, at least in part, reflect a metabolic regulation mechanism during the early stage of natural hibernation when food is absent.

In summary, we uncover a mechanism of deep metabolic suppression (Fig. 7E) and describe a procedure for controlling suspended animation of nonhibernating mammals, including mice, rats, and dogs. The underlying mechanism of uric-acid-regulated metabolic suppression in T2DM is similar to that of natural hibernation. While humans are nonhibernators, the ability to undertake suspended animation could be preserved. Currently, mild hypothermia is an important clinical tool since cells have reduced metabolic activity and will survive longer under hypoxia stress (56). Potentially, severe hypothermia, as described here, could produce additional protection from hypoxia stress.

**Experimental procedures**

**Experimental animals**

We used male, 10–12-week-old C57BL/6 wild-type mice, ob/ob diabetic, and nondiabetic mice, C57BL/Ks db/db diabetic mice, and HFD-STZ-induced T2DM mice. Male adenosine receptor A1−/−, A2a−/−, A2b−/−, and A3−/− mice aged 8–10 weeks were also used in this study. Male Sprague-Dawley rats were between 8 and 10 weeks old. Four Beagle dogs (two male/two female) aged 0.8–1 year and weighing 6.8–10 kg were included. Six pigs (three male/three female) aged 5–6 months weighing 15–20 kg were used. All animals were housed in a standard animal facility (ambient temperature, 22 °C–25 °C; relative humidity, 40%–60%) under a 12-h/12-h light/dark cycle and provided with standardized food and water. Mice or rats were housed two per cage unless otherwise specified. Beagle dogs or pigs were group-housed dependent on gender. All animal care and use procedures were approved by Institutional Animal Care and Use Committee at Nanjing University of Science and Technology (ACUC-NUST-20180012).

**HLSA induction**

For mice and rats, each animal was placed in an individual precooled cage with standard chow and water in a
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temperature-controlled chamber (LRH-500CL, Shanghai Yiheng Scientific Instrument Co, Ltd; 0 °C for type 2 diabetic mice and hyperuricemic mice, 4 °C for drug-induced nondiabetic mice and rats). The chamber temperature was immediately set at an appropriate temperature (17 °C for type 2 diabetic mice, 15 °C for nondiabetic mice or rats; heating rate: 8 °C/min) once the animals were losing righting reflex. For the recovery stage, animals can arouse spontaneously or under warm conditions (32 °C for type 2 diabetic mice, 25 °C for mice; heating rate: 8 °C/min). For beagles and pigs, each animal was first placed in an individual cage in a temperature-controlled ice room (4 °C). Once the beagles were losing righting reflex, the room temperature was immediately set at 10 °C. For the recovery of beagles, animals were removed to a warm animal facility (25–30 °C). Specially, the whole trunk hairs of rats and beagles were removed by electrical shaving with isoflurane anesthesia 2 days prior to the experiments. The shaved rats and beagles were remained on a heating pad and returned to their standard animal facility only after full recovery of body temperature and motor activity. All experiments started at about 8:00 AM and were performed under light illumination. Unsacrificed dogs and pigs were returned to the animal center to continue breeding.

Hyperuricemic model establishment

The mice hyperuricemic models were established as described previously (57). Briefly, C57BL/6 mice were injected intraperitoneally with potassium oxonate (Sigma, catalog no. 156124, 100 mg/kg) and inoculated orally with hypoxanthine (Sigma, catalog no. H9377, 600 mg/kg) at meantime for 7 consecutive days. HLSA induction was performed on the eighth day.

Measurements of core body temperature, heart rate, and respiratory rate

Core body temperatures were recorded with a rectal probe connected to a digital thermometer (BAT-12, Microprobe-Thermometer, Physitemp, NJ, US). Infrared images were recorded with an infrared camera (HT-18, Handheld-Infrared-Thermal-Imaging-Camera, Hit). Heart rates and respiratory rates were monitored via the Small Animal Physiological Monitoring System (75–1500, Harvard Apparatus Inc).

Measurements of locomotor activity and oxygen consumption

Locomotor activity and oxygen consumption were monitored using four VersaMax Animal Activity Monitors (AccuScan Instruments) combined with Fusion Metabolic System (AccuScan Instruments), each consisting of a Plexiglas chamber (40 cm × 40 cm × 30.5 cm). Plexiglas chambers were placed in a temperature-controlled chamber (LRH-500CL, Shanghai Yiheng Scientific Instrument Co, Ltd). A constant airflow (0.5 l/min) was drawn through the chamber and monitored using a metabolic monitor (AccuScan Instruments). The oxygen consumption rate was assessed at 10-min intervals.

Drug administration

ATP disodium salt hydrate (Sigma, catalog no. A1852), 5’-AMP sodium salt (Sigma, catalog no. A1752), and adenosine (Sigma, catalog no. A9251) were administered by intraperitoneal injection (1 μmol in PBS/g body weight). To study the role of P2X and P2Y receptor activation in ATP-induced HLSA, Suramin sodium salt (MCE, catalog no. HY-B0879A) and PPADS tetrasodium (MCE, catalog no. HY-101044) were injected intraperitoneally 1 h prior to ATP treatment. (suramin sodium salt, 75 mmol in PBS/kg; PPADS tetrasodium, 140 mmol in PBS/kg). To study the role of uric acid accumulation in HLSA, allopurinol (MCE, catalog no. HY-B0219, 20 mg/kg) or febuxostat (Beyotime Biotechnology, catalog no. SF1114, 5 mg/kg) was injected intraperitoneally 1 h prior to ATP treatment. For db/db diabetic mice, allopurinol (20 mg/kg) or febuxostat (5 mg/kg) was injected intraperitoneally for 4 consecutive days, then exposed to the cold 1 h after the last injection. Allopurinol and febuxostat were dissolved in DMSO, diluted in sterile saline to a final value of DMSO <10%. To study whether the inhibitor of NHE1 can induce HLSA, zoniporide hydrochloride hydrate (MCE, catalog no. HY-105064D, 4 mg/kg) was injected via the tail vein, and then mice were exposed to cold.

Tissue and blood sampling

Mice were euthanized by cervical dislocation. Blood was collected from the carotid arteries in anticoagulant tubes containing a stop solution (0.2 mmol/l dipyridamole, 5 mmol/l erythro-9-(2-hydroxy-3-nonyl)-adenine, and 4.2 mmol/l EDTA) (58). Then organs were removed and freeze-clamped in liquid nitrogen within 5 s. Blood samples were immediately centrifuged at 3000g for 5 min at 20 °C. The plasma samples obtained were and then stored on ice and immediately used in the experiments.

HPLC analysis of nucleotides and the metabolites

Mouse organs and the plasma samples were homogenized using ice-cold 0.4 mol/l perchloric acid. Samples were analyzed using HPLC (Waters 1525 System; Millipore) on a reversed-phase C18 column as described previously (58, 59). The mobile phases are Buffer A (150 mM KH2PO4, 150 mM KCl, pH 6.0) and Buffer B (150 mM KH2PO4, 150 mM KCl, 15% acetonitrile, pH 6.0). Pure nucleotides and metabolites were used to identify the peaks and obtain the calibration curves. Nucleotides and their metabolites were quantitated based on the peak area compared with a standard curve and normalized to the protein content of frozen tissues.
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1% penicillin-streptomycin under humidified and 5% CO₂ conditions.

Glucose uptake

Glucose uptake assay was performed by using 2-NBDG (60). Briefly, Aml12 cells seeded in 24-well were treated with ATP. Then the medium was removed. The wells were added 2-NBDG (100 mg/ml) in a serum-free low glucose DMEM medium for 20 min at 37 °C. The cells were examined, and photographs were obtained with a fluorescence microscope (Nikon). The fluorescence intensity of 2-NBDG was recorded in the FL1 channel by using a FACScan laser flow cytometer (NovoCyte). Data from 10,000 single-cell events were collected.

Intracellular pH measurement

The measurements of intracellular pH (pHᵢ) were performed as previously described, with modification (61). AML12 cells were used and stained with Hanks' buffered saline solution (HBSS) solution containing 1 μmol/l (2',7'-bis-[carboxyethyl]-5-[and-6]-carboxyfluorescein)-tetraoctoxymethyl ester (BCECF-AM) for 20 min at 37 °C and then rinsing the cells three times with dye-free solution. Then the cells were perfused with HBSS, and chemicals were added via a whole chamber perfusion system (KSX-Type1, Tokai Hit Co). pHᵢ measurements were performed at 37 °C using a fluorescence microscope (Nikon). BCECF fluorescence was excited at 490 nm and 440 nm, and emitted fluorescence was measured at 530 nm. The F490/F440 emission ratio was converted to a pH scale according to the nigericin technique.

Detection of Na+/H+ exchanger activity

AML12 cells were acidified with 20 mmol/l propionate replacing 20 mmol/l NaCl (62). The solutions used in the Na+/H+ exchanger activity measurements were composed of (in mM) as described earlier: NaCl 145, K₂HPO₄ 1.6, KH₂PO₄ 0.4, Ca-glucolactone 1.3, MgCl₂ 1, and D-glucose 5; pH was adjusted to 7.4.

GK and PFK activity assays

GK and PFK activities in the liver extract were determined using a spectrophotometric enzymatic cycling assay kits (Solarbio) according to the manufacturer’s instructions.

Homology modeling and docking

Human NHE1 (uniprot P19634, gene SLC9A1) secondary structure and transmembrane domain topology were predicted using Phyre2 homology modeling portal (http://www.sbg.bio.ic.ac.uk/phyre2) as described previously (63). The resulting model of NHE1 with PDB 4CZB as a template comprised amino acids 100–500, which is the transmembrane domain that contains 12 transmembrane helices. Autodock software (AutoDock 4.2) was used to perform protein compound docking analysis. Docking grid boxes were first set as the complete model of the NHE protein. Docking results indicate that UA is bound inside the Na⁺ binding pocket. The compounds were redocked with the specific binding to the Na⁺-binding pocket in order to achieve a more precise docking result. Molecular docking results were illustrated using the PyMol molecular graphic program.

mCherry-NHE1 constructs and fragment knockout

The sequence for the human NHE1 (uniprot P19634, gene SLC9A1) protein was obtained from Uniprot. DNA sequence was cloned into the pmCherry-N1 vector using the appropriate restriction sites with kanamycin resistance genes. The knockout of the possible UA-binding domain (15 amino acid residues from position 156–170) was generated by overlap extension PCR. The PCR primers were as follows: NHE1P1-F-EcoRI: GAA TTC GCC ACC ATG GTG CTG CGC A; NHE1P1-R: TAG CCG GCC TCC AGA AGA TTA TIC TGG TCG CCC ACC CCC TTG ATG A; NHE1P2-F: TCA TCA AGG GCG TGG GCG AGA CAC CTA TTA TIC TGG AGC CCG GCT A; NHE1P2-R-BamHI: TTA AGG ATC CCG AGA GCC TCC TCC. Mutant constructs were confirmed by DNA sequence analysis.

Membrane protein preparation and MST analysis

pmCherry-N1 plasmids encoding NHE1 or NHE1 mutant were transfected into NIH3T3 cells using Lip2000 transfection kit (Invitrogen) according to the manufacture’s protocol. Transfected cells were harvested 48 h. The cells were homogenized in PBS buffer containing 1% Triton X-100. The homogenate was then centrifuged at 100,000 x g at 4 °C for 1 h. The supernatant was collected and centrifuged again at 100,000 x g at 4 °C for 1 h. The final pellet was resuspended in MST buffer dissolved with UA. After a short incubation, the samples were loaded into MST standard treated glass capillaries for MST analysis as described above. Each ligand-binding curve was measured at least three independently pipetted measurements.

NHE1 gene silencing and rescue

To knock down endogenous NHE1, Aml12 cells were transfected with siRNA-NHE1 using Lipofectamine 2000 agent (Invitrogen) as recommended by the manufacturer’s guidelines. NHE1 knockdown was rescued by cotransfecting of the pcDNA3.1 plasmid carrying a sequence-optimized NHE1-WT or NHE1-mutant gene. Briefly, Aml12 cells were cultured in 35 mm dishes (3 x 10⁴ cells). Before transfection, cells were washed three times with PBS to remove any residual serum, and the medium was replaced with Opti-MEM (Gibco). siRNA or pcDNA3.1 was added in Opti-MEM and then mixed with Opti-MEM containing Lipofectamine 2000. The transfection complexes were directly added to each well. After 6 h of incubation, the medium containing transfection complexes was removed and changed with a fresh medium. Cells were further...
incubated for 48 h and stained for intracellular pH measurement.

**Plasma biochemistry and cytokine assay**

Plasma CK-MB, creatinine, BUN, AST, and ALT activities were measured by a spectrophotometric enzyme cycle assay kit (jiancheng) according to the manufacturer’s instructions. Serum MMP-1, IL-1β, and CRP levels were measured by enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology Ltd) according to the manufacturer’s protocol.

1H NMR spectra of sample extracts were corrected for phase and baseline distortion and referenced manually by the same person to the TSP resonance at δ 0.00 using the TOPSPIN (V3.0, Bruker Biospin). Spectral regions δ 0.50–9.50 were automatically binned using a dynamic adaptive binning approach with equal width of 0.005 ppm. The noisy and residual water-affected regions were removed. The remaining spectral data was normalized by probabilistic quotient normalization (PQN) prior to pattern recognition analysis (65).

**Flow cytometry**

Flow cytometry was performed on draining lymph nodes and spleen. Single-cell suspensions were isolated from spleens and draining lymph nodes. Briefly, after underwent ATP-induced hibernation-like state once a day for 10 consecutive days, mice were sacrificed, and the whole spleen (about 80 mg) and draining lymph nodes (about 10 mg) were ground into small pieces, homogenized by a plastic pestle, and minced through sterilized meshes (200 meshes). Erythrocytes were lysed in a red blood cell lysis buffer (hypotonic ammonium chloride buffer). Approximately 100,000 harvested cells from draining lymph nodes and spleen. Conjugated monoclonal antibodies to mouse FITC rat anti-mouse CD4 Clone GK1.5 (eBioscience Cat#: 11-0041-82), PerCP-Cyanine5.5 rat anti-mouse CD8 Clone 53-6.7 (eBioscience Cat#: 45-0081-80), FITC rat anti-mouse CD11b Clone M1/70 (eBioscience Cat#: 17-0112-82), PE rat anti-mouse CD44 Clone IM7 (eBioscience Cat#: 12-0441-82), APC rat anti-mouse CD62 L Clone MEL-14 (Biolegend Cat#: 104412), FITC rat anti-mouse B220 Clone RA3-6B2 (eBioscience Cat#: 11-0452-82) were used. The analysis was performed by a NovoCyte flow cytometer (ACEA Bioscience Inc).

**Statistical analysis**

Statistical analysis between groups was performed as the following: p values were calculated using one or two-tailed Student’s t test for pairwise comparison of variables; one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons of variables; two-way ANOVA followed by Tukey’s post hoc test for multiple comparisons involving two independent variables. p values: *p < 0.05; **p < 0.01. All results with p < 0.05 were considered statistically significant. Sample sizes of all experiments were pre-determined by calculations derived from our experience. No sample was excluded from the analyses. Animals were not randomly assigned during collection, but the strain, sex, and age of the mice were the same, and the data analysis was single masked. Investigators were not blinded to the group allocation during the experiment and outcome assessment. The number of replicates was indicated in each figure legend. The mean of the technical replicates was used per biological replicate. All statistical tests were justified as appropriate, and the data met the assumptions of the tests. There was an estimate of variation within each group of data.

**Data availability**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jianfa Zhang (jfzhang@mail.njust.edu.cn). This study did not generate new unique materials. All data are contained within the article.

**Supporting information**—This article contains supporting information.

**Author contributions**—Yang Zhao, R. C., Yue Zhao, W. G., Y. Y., Z. D., X. X., Z. Wang, and J. Z., conceptualization; Yang Zhao, Yue Zhao, W. G., Y. Y., Z. D., and J. Z., data curation; Yang Zhao, R. C., Y. Y., X. X., Z. Wang, and Z. Wu, formal analysis; J. Z., funding acquisition; Yang Zhao, Yue Zhao, and W. G., investigation; Yang Zhao, R. C., Yue Zhao, W. G., Y. Y., Z. D., X. X., Z. Wang, Z. Wu, and J. Z., methodology; J. Z., project administration; R. C., Yue Zhao, X. X., Z. Wang, Z. Wu, and J. Z. resources; Yang Zhao, Yue Zhao, W. G., Y. Y., Z. D., X. X., and Z. Wu, software; R. C., Z. Wang, Z. Wu, and J. Z., supervision; Yang Zhao, R. C., Yue Zhao, W. G., Y. Y., Z. D., X. X., Z. Wang, and J. Z. validation; Yang Zhao, R. C., Yue Zhao, W. G., Y. Y., Z. D., X. X., Z. Wang, and Z. Wu, visualization; Yang Zhao, R. C., and J. Z., writing—original draft; Yang Zhao, R. C., Yue Zhao, W. G., Y. Y., Z. D., X. X., Z. Wang, Z. Wu, and J. Z., writing—review and editing.

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**Abbreviations**—The abbreviations used are: A1, A1 adenosine receptor; A2a, A2a adenosine receptor; A2b, A2b adenosine receptor; A3, A3 adenosine receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK-MB, creatine kinase-MB; CRP, C-reactive protein; GK, glucokinase; IL-1β, interleukin-1; MMP-1, matrix metalloproteinase-1; OPLS-DA, orthogonal projections to latent structures; PCA, principal components analysis; PFK, phosphofructokinase; pHic, intracellular pH;
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SD, standard deviation; \(T_{\text{air}}\), ambient temperature; \(T_{\text{cb}}\), core body temperature.

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