Behavioural Brain Research (2007) 178(1):115-122.

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BRAIN ECF ANTIOXIDANT INTERACTIONS IN HAMSTERS DURING AROUSAL FROM HIBERNATION

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Pages 35, Figures 3

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Running title: ECF antioxidants during arousal

Key words: microdialysis; free radicals; torpor; metabolism; striatum

ABSTRACT

Warming from hibernation to cenothermia involves intense metabolic activity and large fluxes in regional blood flow and volume. During this transition, levels of the antioxidants, ascorbate (AA), urate and glutathione (GSH) in brain tissue, extracellular fluid (ECF) and plasma change substantially. Striatal ECF was sampled and manipulated using very slow perfusion microdialysis to examine the mechanisms that influence the changing profile of striatal ECF AA, urate and GSH levels during arousal from hibernation to cenothermia in Syrian hamsters (*Mesocricetus auratus*). Omission of glucose from the perfusate had no effect upon the respective decrease, increase and transient increase in striatal ECF levels of AA, GSH and urate observed during arousal from hibernation to cenothermia. In contrast, inhibition of xanthine dehydrogenase/oxidase (XOR) activity by reverse dialysis with oxypurinol, itself a free radical scavenger, decreased ECF urate and preserved ECF AA levels. This suggests that some ECF AA is oxidized by free radical products of XOR flux and/or by other free radical producing processes activated during the transition from hibernation to cenothermia. Local supplementation of ECF AA, GSH and cystiene had no effect upon the profile of transient increase of ECF urate observed during arousal from hibernation. The production of free radicals by XOR and the disappearance of AA from the ECF continues for at least 2 h immediately after the hamster has attained cenothermia. The hamster, immediately after arousal from hibernation, can be utilized as a natural model to study free radical production and effective scavenging at cenothermia.

INTRODUCTION

In the brain, water-soluble non-enzymatic antioxidants, such as ascorbate (AA), urate and glutathione (GSH), have tissue specific distributions [9,35], are homeostatically regulated, and are required for maintaining the cellular reducing environment which is essential for optimal activity of most enzymes [25]. In addition to having a role as cofactors for many enzymes they also have a direct and indirect role as cytoprotectants during oxidative stress [42]. In brain extracellular fluid (ECF), antioxidant levels are regulated by a combination of transporter-dependent uptake from, and release into blood, and release into ECF from local neuronal and non-neuronal synthesis [9,24,35]. Changes of large magnitude in antioxidant levels in brain tissue [6,15] and ECF [10] are only recorded in response to experimentally induced pathology such as cerebral ischemic and reperfusion although chronic antioxidant tissue deficiency or free radical production in excess of cellular antioxidant capacity is considered to play a role in the eitiology of some neurological diseases [25,37].

Mammalian hibernation is a naturally induced metabolic depression and re-warming from the low body temperatures of hibernation, typically 4–5 ◦C, to cenothermia (IUPS term replacing euthermia, typically 36–37 ◦C) involves the endogenous generation of heat by metabolic activity [30,39] and the induction and later abolition of regional body heterothermy via vasoconstrictive regulation of regional blood flow [33]. This combination of increasing metabolic activity coincident with large fluxes in blood flow and temperature that occurs during arousal from hibernation is similar in some respects to postischemic re-perfusion. The increasing body of evidence

obtained from autopsy and hematological studies demonstrating large changes in tissue and plasma levels of antioxidants [16,39] and hydrogen peroxide and superoxide consuming enzyme activities [3,28,29] between hibernation and cenothermia suggests that hibernators employ a variety of strategies as part of their natural protective responses to the oxidative stress associated with arousal from hibernation.

In order to more completely understand the fate of watersoluble non-enzymatic antioxidants in arousal from hibernation, we recently refined and applied the technique of very slowperfusion microdialysis to monitor the time course of changes in brain ECF levels of AA, urate andGSHin hamsters during hibernation and arousal to cenothermia [31]. This new approach demonstrated that the ECF is a dynamic cerebral compartment during arousal to cenothermia with the levels of AA, urate and GSH changing substantially in the absence of pathology [31]. This current study confirms our previous findings and is the first to utilize reverse dialysis to examine the mechanisms that influence the changing profile of striatal ECF AA, urate and GSH levels during arousal from hibernation to cenothermia.

The first experiment of this series dialyzed the striatum with artificial CSF and measured the profile of changes in striatal ECF AA, urate and GSH that occur during arousal from hibernation. The second experiment tested if local depletion of striatal ECF glucose by dialysis with glucose free CSF would influence the profile of changes in striatal ECF AA, urate and GSH that occur during arousal from hibernation since free radical generation *in vivo* appears to be linked to the depletion of local energy or oxygen reserves, as occurs modestly in exercise [11,23] and excessively in ischemia [35,42].

The third experiment tested if reverse dialysis of the xanthine oxidoreductase (XOR) inhibitor, oxypurinol, would influence the profile of striatal ECF antioxidants during arousal from hibernation. XOR catalyzes the oxidation of hypoxanthine and xanthine to hydrogen peroxide, superoxide and urate subsequent to ATP catabolism [2]. The fourth experiment tested if supplementation of AA, GSH and cysteine to the striatal ECF would influence the profile of striatal ECF urate during arousal from hibernation since ECF GSH levels are low during hibernation and early arousal [31]. These antioxidants were included as supplements since cysteine is the rate-limiting precursor of GSH biosynthesis and GSH exerts its antioxidant activity in synergy with AA [20]. A fifth experiment determined the effect of reverse dialysis of oxypurinol in hamsters cenothermic for 13–16 h after arousal from hibernation on striatal ECF concentrations of AA, urate and GSH. This enabled a comparison between hamsters that were cenothermic for 13–16 h and hamsters that were cenothermic for 1–2 h after arousal from hibernation.

Since hamsters arouse from hibernation at different rates, heart rate (HR)was the principal physiological parameter used to standardize the timing of the onset of the microdialysis sampling periods during arousal from hibernation.

MATERIALS AND METHODS

Animals and housing

The following experiments conformed to the ethical guidelines of the Japanese Physiological Society and Asahikawa Medical University (ethics approval # 06004) and all efforts were made to minimize the number of animals used in the course of this study.

Male and female hamsters (*Mesocricetus auratus*) provided with *ad lib* food and water were housed in darkness at an ambient temperature of 4 ◦C. After approximately 2 months hamsters began their hibernation season. Electrical output from infrared movement and temperature sensors, positioned above and below the hamsters nest, was used to calculated the timing and duration of each hibernation bout. Regularly hibernating animalswere chosen for surgery and experiments [31].

Animal surgery

All surgical procedures were performed on cenothermic hamsters (between 115 and 90 g) under pentobarbital (60 mg/kg IP) (Dainichi Pharmaceutical, Japan) anesthesia. Using an established surgical procedure [31], an ethylene oxide sterilized microdialysis guide tube (BAS MBR-5) was stereotaxically positioned above the striatum (L = 3.0, AP = 0.2mm from bregma, D=− 1.7 from dura, at an angle of 20◦ from flat skull between bregma and lamba) and secured permanently with screws and dental cement. The skin overlaying the latissimus dorsi muscle was pierced bilaterally for the permanent placement of silver rings from which electrocardiograms (ECG) were recorded. After surgery, the incisions were irrigated with xylocaine (AstraZenica, Japan) and dilute penicillin G-potassium solution (Meiji Pharmaceutical, Japan) and each hamsterwas given a 10 mL/kg IP injection of sterile saline. Hamsters re-commenced their hibernation bouts within 2 weeks of surgery.

Very slow flow microdialysis

Brain microdialysis samples the ECF and can be used to simultaneously deliver substances to the ECF by reverse dialysis against an artificial cerebrospinal fluid

(art-CSF) [40]. At very slow perfusion speeds the concentration of diffusible substances in the perfusate will equal their concentration in the ECF [31]. Very slow perfusion microdialysis experiments were performed at 3.5 L/h using a microperfusion pump (WPI 2501, USA). Perfusates were contained in 50 L glass gas tight syringes (SGE, Australia). *In vitro* and *in vivo* experiments used microdialysis probes with 4mm membranes (OD 250 m, MBR 4–5, BAS, USA) perfused at 3.5 L/h with 0.22 m filtered art-CSF (NaCl = 147 mM,KCl = 2.9 mM,MgCl2 = 0.8 mM,CaCl2 = 1.1mMand glucose 1.2mM) or experimental art-CSF solutions as per experimental design. *In vitro* recoveries at 4 ◦C of art-CSF containing 40 M urate, 1mM glucose, 40 M AA and 6 M GSH were 97% for urate, 95% for glucose and AA and 85% for GSH.

For every *in vivo* experiment, newtubingwas used to connect the syringe and the inlet of the microdialysis probe. The hamster cage was placed on a rotatable stage that obviated the need for a liquid swivel in the perfusion line. Dialysates (4 L) were collected into a semi-closed vessel containing 5 L of 0.1M HCl. The dead volume from dialysis membrane to the collection vessel was 0.85 L and this volume was discarded prior to the dialysate collection. The ambient temperature was 4 ◦C. Dialysates were frozen at − 80 ◦C within 2 min of the end of the collection period and only thawed immediately before HPLC analysis.

Hamsters will not enter hibernation when connected to a microdialysis probe. This dictated that the microdialysis probes were permanently inserted into striatal guide tubes of hibernating hamsters. At this time, the microdialysis probes were briefly perfused with art-CSF for about 30 min to remove cellular debris before the inlet and

outlet of the probe was sealed. Handling, insertion and perfusion of microdialysis probes stimulated the hamsters to arouse from hibernation. Hamsters then re-entered hibernation within 11±0.6 h of the insertion of microdialysis probes (n = 16 recordings). Experimental manipulations of striatal perfusate were performed on hamsters during one or two consecutive 60–80 h bouts of hibernation and arousal to cenothermia after insertion of the microdialysis probe. (Hamsters that received oxypurinol were only tested on the first bout of hibernation.) Previous experiments have demonstrated that the profile of changes in striatal ECF levels of AA, GSH and urate sampled by very slow flow microdialysis are not different between the first and second bouts of hibernation [31]. The cenothermic period between these two successive bouts of hibernation was 15±2 h (n = 10 recordings).

During hibernation and arousal, ECG was measured from the implanted silver rings and HR was recorded on a biophysical amplifier (AVB-10, Nihon Kohden, Japan). Cheek pouch temperature was monitored by a thermocouple placed in the cheek pouch as part of the tactile stimulation used to induce arousal from hibernation. Recording of HR and cheek pouch temperatures ceased at the onset of the shivering thermogenesis period before hamsters became cognizant of their surroundings.

All experiments during hibernation and arousal conformed to the following dialysis protocol. After 20 h of hibernation, the inlet of the microdialysis probe was connected to the microsyringe and 2 L of art-CSF was advanced into the probe at 1 L/min. At 10:00 h the following day the probe was perfused at 3.5 L/h and a 4 L dialysate was collected. This represented a no perfusion interval of 16–24 h. This

dialysate was analyzed but is not included in this study in order to standardize the no perfusion interval between dialysate samples. Twenty-four hours later at 10:00 h the following day, a 4 L hibernating dialysate was collected from all hibernating hamsters. This represented a no perfusion interval of 24 h. The perfusate was then changed for the test perfusate and advanced into the dialysis probe by perfusion of 2 L at 1 L/min. Hibernation was confirmed by an unchanged heart rate of about 8 b/min and after 30 min the hibernating hamster was stimulated to arouse by placement of the cheek pouch thermocouple. When the HR of the arousing hamster reached 18 b/min the collection of a 4 L dialysate, encompassing the period of nonshivering thermogenesis (NST) was initiated. During collection of the NST dialysate, hamster mouth temperature increased from 5.1±0.1 to 7.9±0.2 ◦C and HR increased from 18 to 38±2 b/min. When the HR of the arousing hamster reached 115 b/min the collection of a 4 L dialysate, encompassing the period of shivering thermogenesis (ST), was initiated. This collection period encompasses the period of maximum metabolic rate. During collection of the shivering thermogenesis dialysate, hamster mouth temperature increased from 14.4±0.2 to 36 ◦C, HR increased from 115 to 400±8 b/min and respiratory rate increased from 60 breaths/min to peak at 140 and then decreased to about 80 breaths/min. Two 4 L dialysates termed cenothermia 1 h and cenothermia 2 h were collected consecutively immediately following the collection of the shivering thermogenesis dialysate.

A series of cenothermic experiments were performed 12 h after the first arousal from hibernation if the hibernating hamster received an oxypurinol perfusion. In other hamsters, cenothermic experiments were performed 12 h after the second arousal from

hibernation. After arousal from hibernation, cenothermic hamsters with food and water in their home cages were placed overnight at 20 ◦C ambient to prevent the hamster from re-initiating another bout of hibernation. The following morning they were returned to the experimental cold room. At this time the hamsters were transiently active and built nests and food cache. Very slow flow microdialysis sampling was performed 1–2 h after their behavior had returned to normal low levels. Dialysates collected during this time were termed cenothermia 13, 14, 15 and 16 h.

After each experiment the art-CSF test solution in the microdialysis probes was removed by flushing with 8 L of art-CSF at 1 L/min and the inlet and outlet tubings were sealed.

Experiment 1.

ECF AA, urate and GSH profiles during hibernation and arousal from hibernation

In eight hamsters the effect of hibernation and arousal to cenothermia on ECF levels of AA, urate and GSH was measured during dialysis with an art-CSF containing 1.2mM glucose. Discontinuous microdialysis sampling was performed exactly as outlined above.

Experiment 2.

Effect of local glucose depletion on ECF AA, urate and GSH profiles during arousal from hibernation to cenothermia

After collection of the dialysis sample during hibernation, in five hamsters the art-CSF containing 1.2 mM glucose was changed so that the art-CSF contained no glucose and the perfusate was advanced into the dialysis probe by perfusion of 2 µL at 1

μL/min. After 30 min, the hibernating hamster was stimulated to arouse by placement of cheek pouch thermocouple and discontinuous microdialysis sampling was performed exactly as outlined above.

Experiment 3.

Effect of reverse dialysis of xanthine oxidase inhibitor, oxypurinol, on striatal ECF AA, urate and GSH profiles during arousal from hibernation to cenothermia

After collection of the dialysis sample during hibernation, in six hamsters the art-CSF containing 1.2 mM glucose was changed to include 60 μM oxypurinol and the perfusate was advanced into the dialysis probe by perfusion of 2 μL at 1 μL/min. After 30 min, discontinuous microdialysis sampling was performed exactly as outlined above.

Experiment 4.

Effect of local supplementation of AA, cysteine and GSH by reverse dialysis on striatal ECF urate profile during arousal from hibernation to cenothermia

After the collection of the dialysis sample during hibernation, in five hamsters the art-CSF containing 1.2mM glucose was changed to include 800 μM AA, 5 μM cysteine and 5 μM GSH. The perfusate was advanced into the dialysis probe by perfusion of 2 μL at 1 μL/min and after 30 min, the hibernating hamster was stimulated to arouse by placement of cheek pouch thermocouple. Discontinuous microdialysis samplingwas performed exactly as outlined above. Reverse dialysate levels of cysteine and GSH are 2–3 times basal striatal ECF concentrations measured in cenothermic hamsters [31]. Reverse dialysate levels of AA are initially more than 10 times higher than basal striatal ECF levels, however AA in CSF is oxidized by oxygen in solution

and at assay dialysate levels were 100 μM, approximately 2–3 times basal striatal ECF levels during hibernation.

Experiment 5.

Microdialysis experiments 13 hr to 16 hr after arousal to cenothermia.

Twelve hours after arousal the hamster was returned to the cold room, the microdialysis probe was connected and flushed with 2 μL of art-CSF at 1 μL/min. Thirty minutes later perfusion with art-CSF at 3.5 μL/h commenced. In one group of six hamsters, four consecutive 4 μL dialysates were collected. In a second group of six hamsters two consecutive 4 μL dialysates were collected. The art-CSF was then changed to include 60 μM oxypurinol and the perfusate was advanced into the dialysis probe by perfusion of 2 μL at 1 μL/min and 30 min later perfusion at 3.5 μL/h commenced and two consecutive 4 μL dialysates were collected.

Bbrain tissue, blood collection and analysis

Brain tissues were collected from four hibernating hamsters (29-62 h) and seven cenothermic hamsters 3 h to 2 weeks after arousing from hibernation. Cenothermic hamsters were anesthetized with pentobarbital before euthanasia. Blood was collected by heart puncture. Plasma was collected after refrigerated centrifugation. All hamsters were killed by decapitation. Dissection and processing of samples was performed at ambient 4 °C. The brain was rapidly removed and dissected. The forebrain ipsilateral to the microdialysis probe was retained for examination and the remainder of the brain was dissected into 3 regions, forebrain, midbrain and cerebellum for tissue analysis. The forebrain region included the cortical lobes, hippocampal and striatal

tissue but not olfactory bulb tissue. The midbrain region included thalamic and hypothamalic tissue. Cerebellum excluded underlying brainstem. Brain tissues were rinsed of blood in ice-cold saline, blotted and weighed. Tissues were immediately frozen in liquid nitrogen and stored at –80 °C until processing for analysis. Frozen tissue was homogenized in 10 volumes by weight of 12 % PCA in a motor driven glass homogenizing tube and then centrifuged at 27,000 x g for 15 minutes. Supernatants were neutralized with 8M KOH and centrifuged at 27,000 x g for 15 minutes to remove $KClO_4$ before HPLC. Plasma was analyzed by HPLC after dilution in 10 parts ice cold HPLC buffer and filtration (0.22 μm Minisart Sartorius, Germany).

HPLC analysis

AA, GSH and urate in standards or dialysate samples (4–5 L) were separated byHPLCusing a Synergi 4 mHydro-RP80Acolumn (150mm x 4.6 mm, Phenomenex, USA) and guard column (Brown Lee) and detected electrochemically at a potential of 1.1V on a carbon working-electrode as per a previously published method [31]. The composition of the mobile phase was 25mM NaH_2PO_4, adjusted to pH 2.5–2.7, with HCl. Data was stored and analyzed on a computer using Chemstation HP.

Verification of MD probe placement

The position of the microdialysis probe in the striatum was verified by microscopic examination of coronal sections as per a previous published protocol [31].

Statistics

All results are presented as means ± SEM. Comparisons of forebrain tissue contents and plasma levels of analytes between animals were by unpaired t-test.

Oneway repeated measures ANOVA with Tukey post-hoc comparisons were used to compare the changes in ECF analyte levels within treatments during arousals from hibernation and cenothermic experiments. Kruskal-Wallis nonparameteric ranking test with Dunn's correction for multiple comparisons was used for comparisons between treatments of the percentage changes at ST, CEN 1 h and CEN 2 h. Statistical significances are presented.

RESULTS

Forebrain, midbrain and cerebellum tissue contents of antioxidants during hibernation and cenothermia

Tissue contents of AA, GSH and urate, from forebrain, midbrain and cerebellum tissue of seven cenothermic hamsters sacrificed 3 h to 2 weeks after arousal from hibernation and of four hibernating hamsters sacrificed after 29–62 h of hibernation is presented in Fig. 1.

Tissue AA content from cenothermic hamsters was higher in forebrain ($P<0.001$) than midbrain or cerebellum ($F(2,31)=28.8$, $P=0.0001$). Hibernation did not change tissue AA content. In these same animals, tissue GSH content was higher in forebrain ($P<0.001$) than in midbrain or cerebellum ($F(2,31)=248$, $P=0.0001$) and regional tissue content was not changed by hibernation. At cenothermia, tissue urate content was lower in cerebellum ($F(2,17)=9.6$, $P=0.002$) than in forebrain ($P<0.001$) or in midbrain ($P<0.01$). Relative to cenothermia, tissue levels of urate in forebrain ($t=5.4$, $df=9$, $P=0.0004$), midbrain ($t=5.7$, $df=9$, $P=0.0003$) and cerebellum ($t=3.8$, $df=9$, $P=0.0007$) were decreased by 65%, 77% and 60% respectively in hibernation.

Plasma levels of antioxidants during hibernation and cenothermia

In cenothermic hamsters 3 h to 2 weeks after arousal to cenothermia, plasma AA was 14.6±0.8 μM (n = 7) while in hibernating hamsters (29–62 h) plasma AA levels were 12.2±0.6 μM (n=6) (t = 2.1, d.f. = 11, P = 0.06). (In a previous paper we mistakenly reported that plasma AA was higher in hibernation [31].) Plasma urate levels were 7.2±0.9 μM(n=7) in cenothermic hamsters and not different in hibernating hamsters 7.4±0.7 μM (n=6) (t = 0.4, d.f. = 11, P = 0.68). Technical problems determined that GSH levels could not be reliably measured from filtered plasma samples.

Striatal ECF levels of analytes during hibernation and arousal to cenothermia

The physiological parameters and time course of changes in striatal ECF AA, urate and GSH during arousal from hibernation to cenothermia and the effect of local manipulation of perfusate upon analytes is shown in Fig. 2.

Ascorbate

Basal striatal ECF dialysate levels of AA during hibernation in animals receiving dialysis with art-CSF was 44.4 ± 3.2 μM (n=24). Basal striatal ECF dialysate levels of AA during hibernation were 49.5 ± 5.8 μM in 8 hamsters continuing to receive art-CSF during arousal, 58.8 ± 3.6 μM in 5 hamsters to receive art-CSF without glucose during arousal, 33 ± 4.2 μM in 6 hamsters to receive art-CSF containing oxypurinol during arousal and 35.5 ± 2.9 μM in 5 hamsters to receive art-CSF containing supplemental antioxidants during arousal. ECF dialysate levels of AA were significantly less in the group to be perfused with oxypurinol containing art-CSF than in hamsters in

the group to be perfused with glucose free art-CSF ($F(3,23)=4.7$, $P=0.02$) ($P<0.05$). For each treatment results are presented as increases or decreases relative to basal striatal ECF dialysate levels of AA during hibernation. Arousal from hibernation to cenothermia was associated with a 400% decrease in dialysate AA in hamsters perfused with art-CSF ($F(4,39)=44.9$, $P<0.0001$) and hamsters perfused with glucose free art-CSF ($F(4,24)=31.4$, $P<0.0001$). Perfusion with art-CSF containing 60 μM oxypurinol reversed the decrease in ECF dialysate AA normally observed during arousal from hibernation to cenothermia ($F(4,29)=1.6$, ns). Relative to striatal ECF dialysate levels of AA measured in hibernation, striatal ECF dialysate levels of AA collected during cenothermia 1h & cenothermia 2h) were significantly higher in hamsters given oxypurinol perfusion than art-CSF perfusion ($KW=7.2$, $P=0.019$)($P<0.05$) Fig. 2A.

Urate

Basal striatal ECF dialysate levels of urate during hibernation were 31 ± 2.8 μM (n=24). Basal striatal ECF dialysate levels of urate during hibernation were 39.8 ± 5.2 μM in 8 hamsters continuing to receive art-CSF during arousal, 36.5 ± 5.3 μM in 5 hamsters to receive art-CSF without glucose during arousal, 20.1 ± 3.0 μM in 6 hamsters to receive art-CSF containing oxypurinol during arousal and 24.5 ± 4.4 μM in 5 hamsters to receive art-CSF containing supplemental antioxidants during arousal. ECF dialysate levels of urate were significantly less in the hamsters in the group to be perfused with oxypurinol containing art-CSF than in the group to be perfused with art-CSF ($F(3,23)=4.1$, $P=0.02$) ($P<0.05$). For each treatment results are presented as

increases or decreases relative to basal striatal ECF dialysate levels of urate during hibernation.

Relative to striatal ECF dialysate levels of urate measured in hibernation, striatal ECF dialysate levels of urate increased 100% during shivering thermogenesis and returned to near hibernation levels during cenothermic 1 h & 2 h when the perfusion medium was art-CSF ($F(4,39)=23.1$, $P=0.0001$), glucose free art-CSF ($F(4,24)=19.0$, $P=0.0001$) and antioxidant supplemented art-CSF ($F(4,24)=10.3$, $P=0.0003$). Perfusion with oxypurinol containing art-CSF significantly attenuated the increase in striatal ECF dialysate levels of urate during shivering thermogenesis and decreased striatal ECF dialysate levels of urate during cenothermic 1 h & 2 h (($F(4,29)=17.1$, $P=0.0001$) ($P<0.05$). Relative to striatal ECF dialysate levels of urate during hibernation, striatal ECF dialysate levels of urate collected during shivering thermogenesis (ST) were significantly lower in hamsters given oxypurinol perfusion than other infusions ($KW=12.7$, $P=0.0053$)($P<0.05$) Fig 2B.

Glutathione

In all groups, basal striatal dialysate GSH levels were below the limit of detection (0.2 μM/3 μL) during hibernation. Basal striatal ECF dialysate levels of GSH at cenothermia were 1.0±0.16 μM (n = 19) and not different between treatments ($F(2, 18) = 0.6$, ns). For each treatment, results are presented as increases or decreases relative to basal striatal ECF dialysate levels of urate during cenothermia. The limit of analytical detection (0.2 μM) was used to approximate ECF levels during hibernation and NST in the graphical presentation of results. Relative to striatal ECF dialysate

levels of GSH during hibernation, striatal dialysate levels of GSH had increased by ST and continued to increase until cenothermia. This profile was not influenced by any perfusate (Fig. 2C).

Effect of reverse microdialysis with oxypurinol during cenothermia 13hr – 16hr after arousal from hibernation

The effect of perfusion of art-CSF or art-CSF containing 60 µM oxypurinol on striatal ECF dialysate levels of AA, urate and GSH in cenothermic hamsters 13–16 h after arousal from hibernation is shown in Fig. 3. Basal striatal ECF levels of AA, urate and GSH were 5.1±0.8 µM (n = 12), 35.2±5.6 µM (n = 12) and 1.7±0.4 µM (n = 12), respectively. There was no difference between basal levels in groups allocated to either perfusion treatment. Perfusion with oxypurinol containing art-CSF decreased striatal ECF levels of urate by 45% ($F(3,23) = 8.0$, $P = 0.002$) (CEN 14 h versus CEN 15 h and CEN 16 h, $P < 0.05$) but did not influence striatal ECF levels of AA ($F(3, 23) = 0.04$, $P = 0.98$) or GSH ($F(3, 23) = 17.1$, $P = 0.08$) ($P < 0.95$).

DISCUSSION

In healthy people and animals at cenothermic temperatures, tissue, ECF and plasma levels of antioxidants are regulated and recycled [9,35] with diminished or abnormal levels being correlated to some diseased states [4], smoking [1] or gross pathology [41]. In contrast, arousal from hibernation in hamsters appears to be a unique period in which large fluxes in the levels of antioxidants in tissues and fluid compartments [28,29,31] are associated with the ATP consuming transition between the vastly different metabolic states of hibernation and cenothermia. These changes in

antioxidant levels presumably reflect adaptive responses to defend against oxidative stress and also the simultaneous influence of other homeostatic chemistries active during this transition.

AA and GSH have non-uniform distribution within the brain, with AA being concentrated in neurons and GSH in glia [35]. Under cenothermic conditions, AA is concentrated from blood into brain ECF via SVCT2 transporters in the choroid plexus [35] and dehydroascorbic acid enters the brain across the blood brain barrier by facilitated diffusion via glucose transporters [8]. In the hibernating hamster, brain tissue content of AA is maintained during hibernation despite 400% higher striatal ECF levels and lower plasma AA levels during hibernation relative to arousal to cenothermia [29] and this paper. (Previously we erroneously reported plasma AA levels to be higher in hibernation [31].) The accumulation of AA in the ECF from blood probably occurs predominantly during the early stages of entry into hibernation but continues at a slow rate during hibernation [31]. During arousal from hibernation, AA is removed from the ECF probably by oxidation as a consequence of free radical scavenging. AA has numerous biochemical roles in addition to action as an antioxidant and the decrease in ECF AA levels during arousal may also result from transport of AA out of the ECF space, possibly for a role in modulation of neurotransmission [35] which returns to normalcy as the brain rewarms.

Brain tissue content of GSH was unaffected by hibernation while ECF GSH which was low during hibernation, began to increase by late arousal and was maximal at cenothermia. The profile and magnitude of increase of ECF GSH over the course of

arousal closely parallels serum GSH levels [28]. The origin of ECF GSH accessed by microdialysis cannot be determined from these experiments but it is considered that the enzymes of GSH synthesis and recycling are intracellular [9,25] and as such it is probable that ECF GSH levels are derived from cerebral intracellular overflow. GSH is a substrate utilized in the scavenging of H_2O_2 by glutathione peroxidase however ECF and serum GSH levels [28] increased as arousal progressed while tissue content was unchanged. This suggests that during arousal the principal role of GSH is for biochemical functions other than H_2O_2 scavenging. The rate of brain tissue GSH turnover, which was not measured, is probably greater at cenothermia than during hibernation and the increased ECF concentrations of GSH in late arousal and cenothermia probably reflects synthesis and recycling [5,21] once a temperature-dependent threshold of approximately 18 °C has been exceeded.

The profile of changes in tissue and ECF urate during arousal from hibernation reflect dynamic components of synthesis, degradation and excretion. Urate, like AA and GSH, appears to have non-uniform distribution within the brain at cenothermia but, in contrast to both AA and GSH, brain tissue content of urate was globally decreased by 65% during hibernation and then replenished within 12 h at cenothermia. Urate, along with H_2O_2 and superoxide, are products of the hydroxylation of hypoxanthine and xanthine catalyzed by XOR, a key intracellular enzyme in purine catabolism. However, a growing body of evidence implicates XOR as having a much broader role in physiology and pathology [2]. Tissue and ultrastuctural distribution of XOR is incompletely determined and species-specific differences in distribution and possibly

physiological function appear likely [7,12,18]. XOR has distinct regional distribution in the rodent brain, with the limited information available indicating that XOR levels are moderate in hippocampus and low to absent in cerebellum [27]. No information is available as to sub-cellular localization of XOR in cerebral tissue. The pattern of XOR distribution in rat appears not to closely correlate with hamster tissue urate levels.

The origin of ECFurate accessed by microdialysis is probably local cerebral synthesis via XOR rather than blood since urate normally exits the brain via organic anion transporters in the apical membrane of the choroid plexus and in capillary endothelial cells [24]. In cenothermic hamsters 15–16 h after arousal from hibernation, striatal ECF urate levels were decreased 45 % by reverse microdialysis with oxypurinol, although no effect upon ECF AA or GSH levels was detected. In rats, semichronic systemic allopurinol administration, which reflects the cumulative effect of allopurinol and oxypurinol, decreased striatal tissue urate content by 36 % [22]. These results suggest that in rodents the brain, like the intestine and the liver [34], maintains chronic purine degradation via XOR under non-ischemic conditions.

At cenothermia, metabolic flux through XOR is increased in response to accelerated catabolism of ATP, as occurs in ethanol consumption [43], exercise in many animal species [11,23] and in conditions of low oxygen tension such as mild ischemia [34]. In hamsters, shivering thermogenesis during arousal from hibernation to cenothermia is associated with peak metabolic rate and cortical blood flow in excess of 250% of basal resting values [30] and elevated brain lactate concentrations [14]. In other species of hibernators, the maximum metabolic rate during arousal from hibernation is recorded

during shivering thermogenesis and is associated with decreased hemoglobin O2 saturation [17] and increased brain tissue lactate [13]. Collectively this suggests that reduced oxygen tension and a period of mild ischemia are routinely tolerated during shivering thermogenesis at each arousal.

During arousal from hibernation, striatal ECF urate levels peak in the late arousal collection that encompasses the period of maximum metabolic rate and shivering thermogenesis. A peak in urate levels at this time is consistent with the established effects of increased metabolism and mild ischemia to enhance purine catabolism and increase the metabolic flux through XOR [11,23,34]. The profile and magnitude of increase of ECF urate over the course of arousal closely parallels serum urate levels in ground squirrels [39]. Reverse dialysis of the XOR inhibitor, oxypurinol, during arousal from hibernation significantly attenuated ECF striatal urate levels during the period of maximum metabolic rate and also in the 2 h of cenothermia immediately following arousal from hibernation. No effect was observed on ECF striatal GSH profile but the decrease of ECF AA levels, normally observed during arousal from hibernation, was attenuated by treatment with oxypurinol. The sparing of AA in response to reverse dialysis with oxypurinol was not observed in hamsters that had been cenothermia for 12 h despite a decrease in ECF urate.

Oxypurinol, in addition to being an inhibitor of XOR, can function as an antioxidant [26] although probably less efficaciously than AA since it has an oxidation potential of 1.0V at physiological pH while that of AA is 0.3V(versus Ag/AgCl/KCl reference electrode). Stoichiometry determines that inhibition of the metabolic flux through XOR, by the action of oxypurinol, decreases the production of not only urate but

also H2O2 and superoxide. Thus the maintenance of high levels of ECF AA by the addition of a non-endogenous free radical scavenger to the ECF and the simultaneous decrease in the production of H2O2 and superoxide provides compelling, although indirect, evidence that the disappearance of some of the AA from the ECF over the course of arousal from hibernation results from oxidation of AA consequence of its functioning as anantioxidant.

In cenothermic rats, the physiological effect of lowering the concentration of glucose in the perfusate on brain physiology is ambiguous. Dialysate glucose levels are reduced by physiological [19] and pharmacological [32] increase of neural activity. However, dialysate neurotransmitter and glycolytic metabolite levels are unaffected by low levels of glucose in the perfusate under hypoxic and normoxic conditions [36]. In contrast, during conditions that induce severe pathology a lack of adequate ECF glucose during hypoxic cardiac arrest contributes markedly to excitotoxic levels of ECF glutamate [38]. In our experiments, very slow perfusion with glucose free CSF had an effect no different from very slow perfusion with CSF containing glucose upon the ECF profile of AA, urate and GSH during arousal from hibernation. This suggests that intracellular energy reserves, that are maintained at high levels in hamsters during arousal from hibernation [14], coupled with diffusion/transport of glucose from the surrounding tissues and blood is sufficient to prevent local metabolic stress induced by local depletion of ECF glucose by perfusion with glucose free CSF during the metabolically demanding transition to cenothermia.

A decrease in ECF urate levels induced by reverse dialysis of the XOR inhibitor, oxypurinol, demonstrates that XOR is functional during arousal and thatH2O2 and superoxide are produced during arousal from hibernation. Our contention was that urate would be consumed in the process of scavenging free radicals during arousal and that the oxidation of urate could be prevented by local supplementation of the ECF with the antioxidants AA, GSH and cysteine by reverse dialysis. However, this treatment had no sparing effect upon ECF urate during arousal suggesting that the free radical species scavenged by AA, GSH, and cysteine are distinct from those scavenged by urate during this transition. Indeed the observation that brain tissue urate content is globally low during hibernation and has increased by cenothermia suggests that it is unlikely that urate functions as a major cellular antioxidant during arousal from hibernation.

The functional significance of the production of free radicals consequent of the enhanced metabolic flux through XOR, which peaks during late arousal from hibernation, is unknown. However, the demonstration that XOR generated superoxide increases basal cortical blood flow (CBF) and impairs cerebral autoregulation [44] may have relevance to the process by which cerebral hemodynamic compliance can be increased to preserve non-pathological integrity during the enormous increase in CBF and loss of autoregulation that hamsters experience late in arousal from hibernation [30] when blood is shunted for warming into the anterior half of the body [33].

It is worth noting that the 2 h period immediately following the attainment of cenothermia after arousal from hibernation, is a temporal window during which the metabolic flux through XOR continues to produce free radicals in the apparent absence of

pathology while AA is oxidized or removed from the brain ECF. It is likely that other free radical generating and scavenging process are also active in hamster tissues at this time. As such the hamster, immediately aroused from hibernation, can be used as a natural model of free radical production and scavenging at *cenothermia*.

Acknowledgements

This work was supported in part by a JSPS Grant-in-Aid for Scientific Research # 18390068 and Exploratory Research # 18650226 to MH. We thank Sharon Hanley for proof reading.

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Figure 1.

Tissue contents of AA, urate and GSH from forebrain, midbrain and cerebellum tissue of seven cenothermic hamsters sacrificed 3 h to 2 weeks after arousal from hibernation (filled histograms) and of four hibernating hamsters sacrificed after 29-62 h of hibernation (open histograms). Mean ± SEM. *** P<0.001; **P<0.01.

Figure 2.

The effect of perfusate on the profile of changes in striatal ECF levels of AA (A), urate (B), GSH (C) during hibernation, arousal from hibernation, and 2h of cenothermia. Profile of changes in heart rate (beats/min) (D) and cheek pouch temperature (°C) (E) during collection of microdialysis samples. All hamsters received perfusion with art-CSF during HIB. Perfusion with art-CSF (solid circles, n = 8 hamsters), glucose free art-CSF (open circles, n = 5 hamsters), 60 μM oxypurinol (solid triangle, n = 6 hamsters) and antioxidant supplemented art-CSF (solid diamond, n = 5 hamsters) during arousal commenced after HIB. Hibernation and non-shivering thermogenesis (NST) dialysates collected from discontinuous sampling. Shivering thermogenesis (ST), cenothermia (CEN 1h and CEN 2h) dialysates collected from continuous sampling. Basal striatal ECF dialysate levels of each analyte are presented in text. Mean ± SEM. SEM are smaller than symbols for heart rate and cheek temperature data. Within treatment, analyte concentration different from HIB *** P<0.001; *P<0.05. Percentage different between treatments at same collection time. Kruskall-Wallis # P<0.05.

Figure 3.

Effect of perfusion of art-CSF (solid circles, n = 6 hamsters or open triangles) or art-CSF containing 60 μM oxypurinol (solid triangles, n = 6 hamsters) on striatal ECF dialysate levels of ascorbate (A), urate (B) and GSH (C) during cenothermia 13 -16h after arousal from hibernation. Basal striatal ECF dialysate levels of each analyte are presented in text. Mean ± SEM. Cen 14h vs other. *P<0.05; **P<0.01.

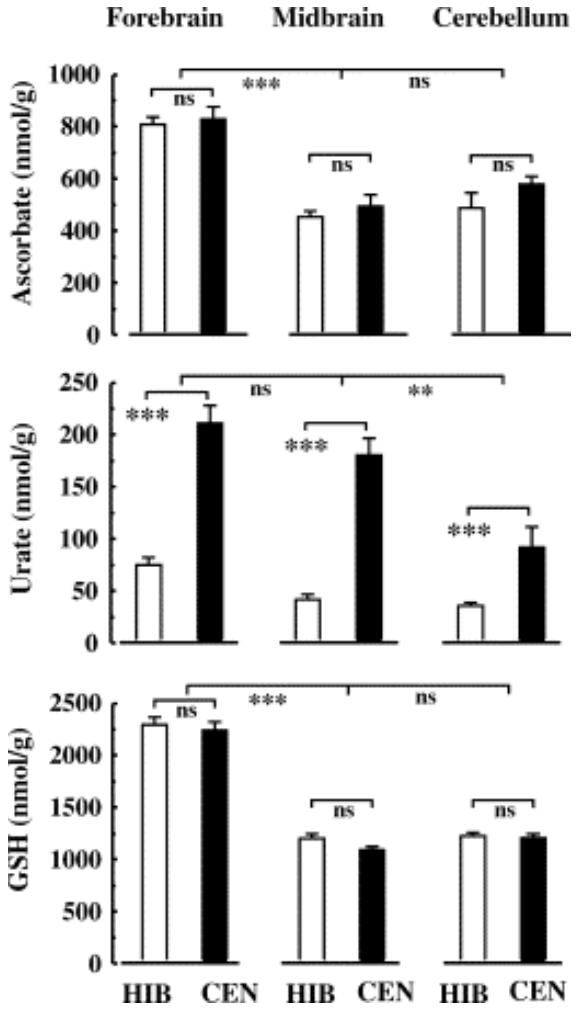
Forebrain
Midbrain
Cerebellum
Ascorbate (nmol/g)
1000
800
600
400
200
0
***
ns
ns
ns
ns
ns
Urate (nmol/g)
250
200
150
100
50
0
ns
**
***
***
***
GSH (nmol/g)
2500
2000
1500
1000
500
0
***
ns
ns
ns
ns
HIB
CEN
HIB
CEN
HIB
CEN

Fig. 1

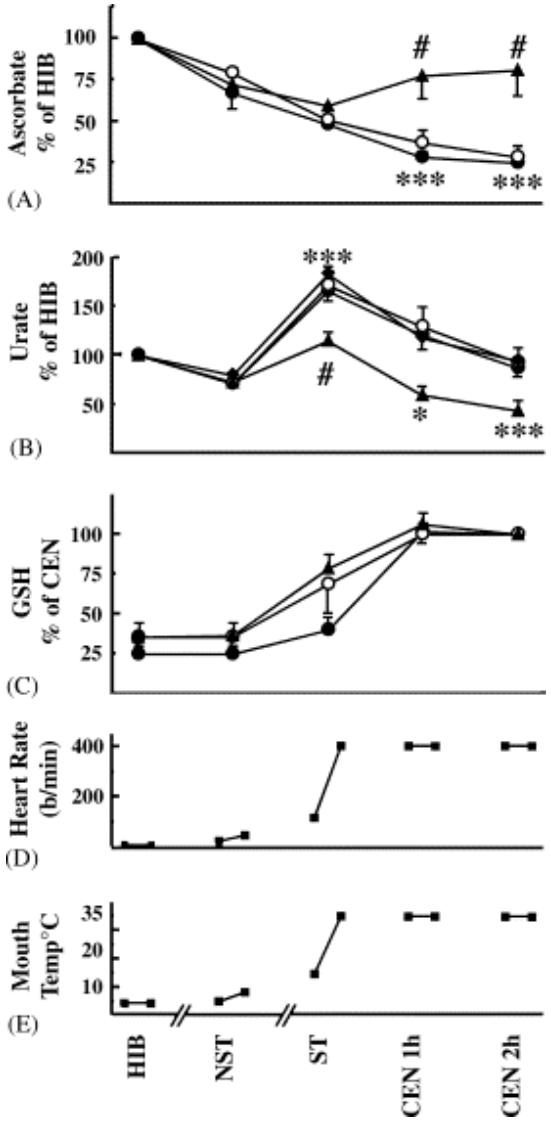
Ascorbate
% of HIB
100
75
50
25
(A)
#
#
***
***
Urate
% of HIB
200
150
100
50
(B)
***
#
*
***
GSH
% of CEN
100
75
50
25
(C)
Heart Rate
(b/min)
400
200
(D)
Mouth
Temp°C
35
20
10
(E)
HIB
NST
ST
CEN 1h
CEN 2h

Fig. 2

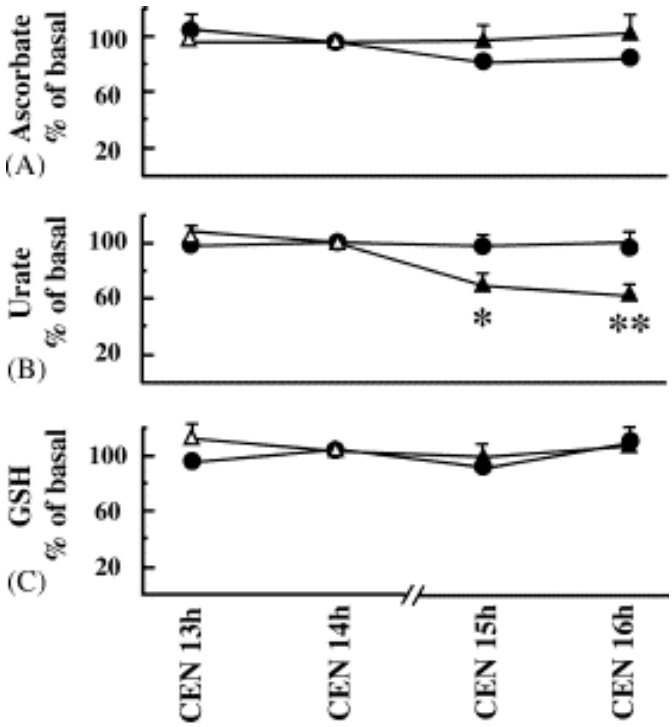
(A) Ascorbate % of basal
100
60
20
(B) Urate % of basal
100
60
20
*
**
(C) GSH % of basal
100
60
20
CEN 13h
CEN 14h
CEN 15h
CEN 16h

Fig. 3