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Original Contribution

Sleep deprivation under sustained hypoxia protects against oxidative stress

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ABSTRACT

We previously showed that total sleep deprivation increased antioxidant responses in several rat brain regions. We also reported that chronic hypoxia enhanced antioxidant responses and increased oxidative stress in rat cerebellum and pons, relative to normoxic conditions. In the current study, we examined the interaction between these two parameters (sleep and hypoxia). We exposed rats to total sleep deprivation under sustained hypoxia (SDSH) and compared changes in antioxidant responses and oxidative stress markers in the neocortex, hippocampus, brainstem, and cerebellum to those in control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). We measured changes in total nitrite levels as an indicator of nitric oxide (NO) production, superoxide dismutase (SOD) activity and total glutathione (GSHt) levels as markers of antioxidant responses, and levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonyls as signs of lipid and protein oxidation products, respectively. We found that acute (6 h) SDSH increased NO production in the hippocampus and increased GSHt levels in the neocortex, brainstem, and cerebellum while decreasing hippocampal lipid oxidation. Additionally, we observed increased hexokinase activity in the neocortex of SDSH rats compared to UCSH rats, suggesting that elevated glucose metabolism may be one potential source of the enhanced free radicals produced in this brain region. We conclude that short-term insomnia under hypoxia may serve as an adaptive response to prevent oxidative stress.
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Sleep deprivation leads to cognitive slowing, memory impairment, decreased vigilance, and diminished sustained attention [1]. It has been hypothesized that free radicals accumulate during waking as a result of enhanced metabolic activity and may be responsible for some of the effects of sleep deprivation [2].

People moving rapidly to high altitude commonly experience acute mountain sickness, pulmonary edema, cerebral edema [3,4], mental dysfunction, memory deficits [5–7], insomnia, dizziness, nausea [8], weight loss [9], and motor impairment [10]. Recent data suggest that humans exposed to high-altitude hypoxia may be at increased risk of oxidative stress [3,11–15]. Increased levels of oxidative stress and neuronal apoptosis have also been reported in animals subjected to hypobaric hypoxia [16–19].

Free radicals, which include reactive nitrogen and reactive oxygen species (RNS and ROS, respectively), are difficult to detect and quantify directly because of their extreme reactivity. The amount of RNS, such as nitric oxide (NO), can be deduced from measurement of the level of its metabolites, nitrates/nitrites (NO_3^-/NO_2^-), whereas the involvement of ROS can be inferred from measurement of antioxidant responses. Antioxidant responses include changes in the activities of several antioxidative enzymes, including superoxide dismutase (SOD), and in the levels of the endogenous antioxidant glutathione (GSHt). If antioxidant responses are unable to successfully scavenge the free radicals, this will lead to oxidative damage to lipids (measured as thiobarbituric acid-reactive substances, TBARS) and/or proteins (measured as protein carbonyls), resulting in oxidative stress [20].

We previously reported that long-term (5–11 days) total sleep deprivation, by the disk-over-water method, decreased SOD activity in the rat hippocampus and brainstem [21]. The rat neocortex did not show any significant changes in SOD or glutathione peroxidase (GPx) activities with either short-term (8 h) or long-term (5–11 days) total sleep deprivation [21,22]. We also previously showed that 6 h of total sleep deprivation increased GPx activity in the rat hippocampus and cerebellum and increased GSHt levels in the neocortex, brain stem, and basal forebrain [23]. On the other hand, D'Almeida et al. [24] reported that 96 h of rapid eye movement (REM) sleep deprivation, by the platform technique, significantly decreased GSHt levels in the rat hypothalamus.

We previously also showed that chronic sustained hypoxia increased the activity of the antioxidative enzyme glutathione reductase

Abbreviations: GSHt, total glutathione; HK, hexokinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDSH, sleep-deprived under sustained hypoxia; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; UCN, undisturbed control under normoxia; UCSH, undisturbed control under sustained hypoxia.

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(GR) in the pons and elevated the level of TBARS in the cerebellum of experimental rats relative to control rats under normoxic conditions [25]. Maiti et al. [16] reported that hypoxia induced oxidative stress in the rat neocortex, hippocampus, and striatum, and Jefferson et al. [13] showed that humans exposed to acute (48 h) or chronic high altitude had increased levels of plasma TBARS and GSHt.

This study was carried out to determine the combined effects of total sleep deprivation and sustained/continuous hypoxia (10% O_2) on antioxidant responses and oxidative stress. This condition is similar to, but not quite the same as, sleep apnea, which is characterized by sleep deprivation/fragmentation under intermittent/cyclic hypoxia (alternating 21 and 10% O_2 [reviewed in 26,27]). We analyzed changes in NO levels, SOD activity, GSHt levels, and levels of TBARS and protein carbonyls in rats subjected to 6 h of total sleep deprivation under sustained hypoxia (SDSH) and compared them to rats left undisturbed under either sustained hypoxia (UCSH) or normoxia/room air (21% O_2; UCN) for the same period of time. We hypothesized that any increase in free radical production could probably result from increased glucose metabolism. Hence we measured the activity of hexokinase (HK), which is the rate-limiting enzyme in glucose metabolism.

Materials and methods

Young adult male Sprague–Dawley rats (400–500 g) were used for all experiments. The experimental protocols were approved by our Institutional Animal Use and Care Committee and conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Hypoxic exposures

Rats were individually housed in commercially designed chambers (30×20×20 in.; Oxycycler Model A44XO; Biospheryx, Redfield, NY, USA). The chambers were operated under a 12-h light/dark cycle (8:00 AM–8:00 PM). On the day of the experiment, the O_2 level was changed from 21 to 10% and the rats were subjected to 6 h of total sleep deprivation beginning at lights on (8:00 AM). Gas was circulated around each of the chambers at 60 L/min (i.e., one complete change every 10 s). The O_2 concentration was continuously measured by an O_2 analyzer and regulated throughout the 6 h of experimental time by a computerized system controlling the gas valve outlets. Deviation from the desired concentration was automatically corrected by addition of N_2 or O_2 through solenoid valves. Ambient temperature was kept at 22–24 °C.

Sleep deprivation habituation

Rats were housed individually in the commercially designed chambers as described above and handled for 1 h each day for 1 week. This gentle handling procedure, as described in our earlier paper [23], included brushing their fur or gently touching them with a blunt-ended wire, introducing objects into their chambers (including paper towels and plastic weigh boats), and disturbing their chamber bedding. These procedures were performed through a small opening in the hypoxic chamber. The chambers were maintained at room air (21% O_2) during the habituation period, and the animals were allowed food and water ad libitum.

Sleep deprivation under sustained hypoxia

After 1 week of habituation to the handling procedure, the animals were divided into three groups: group 1 rats were left undisturbed under room air (21% O_2, unhandled control under normoxia, UCN); group 2 rats were subjected to sustained hypoxia (10% O_2) beginning at lights on, on the day of the experiment (8:00 AM), and were left undisturbed (unhandled control under sustained hypoxia, UCSH); group 3 rats were subjected to sustained hypoxia (10% O_2) beginning at lights on, on the day of the experiment (8:00 AM), and were handled each time they showed physical signs of sleepiness (sleep deprived under sustained hypoxia, SDSH). Our sleep deprivation procedure is based on visual observation of the rat. Many investigators have used the gentle handling procedure to induce short-term sleep deprivation based on behavioral signs of sleepiness [28–30]. At the end of the experimental period (6 h), the rats were sacrificed by decapitation after halothane anesthesia, and the neocortex, hippocampus, brainstem, and cerebellum were dissected on ice and stored at −80 °C until analyzed [23]. The level of NO, the activity of SOD, and the level of GSHt, as well as the levels of TBARS and protein carbonyls and the activity of HK, were analyzed as described below.

Biochemical analysis

Each brain region was divided into two portions; one portion was homogenized in a hand-held homogenizer with 20 strokes in cold homogenizing buffer (50 mM Tris–HCl, pH 7.5, 50 mM $MgCl_2$, and 5 mM EDTA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) to make a 10% homogenate (w/v). The homogenate was centrifuged in an Eppendorf microcentrifuge (5415 C) at 2,000 rpm (320 *g*) for 10 min at 4 °C. The pellet was discarded and approximately 300 μl of the supernatant was used for determining NO levels and HK activity. The remaining supernatant was recentrifuged at 13,500 rpm (14,000 *g*) for 30 min at 4 °C and used for determining SOD activity and GSHt levels. The remaining portion of each brain region was homogenized with 20 strokes in cold homogenizing buffer containing 20 mM phosphate buffer (pH 7.4) to which 1% 0.5 M butylated hydroxytoluene had been added to prevent oxidation during sample preparation. The homogenate (10%, w/v) was centrifuged in an Eppendorf microcentrifuge at 6,000 rpm (2900 *g*) for 10 min at 4 °C. The pellet was discarded and the supernatant was used for determining the levels of TBARS and protein carbonyls.

The protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine plasma γ-globulin as the standard. The amount of protein in the standards and samples was determined on a microtiter plate reader (Molecular Devices Emax precision microplate reader) at a wavelength of 750 nm.

Nitric oxide assay

NO production in situ is difficult to detect because of the rapid decay (within seconds) of this compound in physiological systems. The best index of total NO production is the measurement of the sum of its metabolites, nitrates and nitrites (NO_x). This is achieved by reduction of nitrates to nitrites by metallic cadmium followed by reaction with the Greiss reagent and spectrophotometric detection [31]. In this study we measured the accumulation of total nitrites (NO_2^-) in the samples using the commercial kit from Oxis International (Bioxytech nitric oxide nonenzymatic assay, Cat. No. 22111 N). After color development at room temperature, the absorbance of the samples was measured on a microplate reader at a wavelength of 540 nm within 20 min. Sodium nitrite ($NaNO_2$) was used as an external standard and the levels of NO in the samples were expressed as nmol nitrites/g tissue.

Superoxide dismutase activity

SOD activity was measured according to the method of Misra and Fridovich [32]. Tissue extract was added to carbonate buffer (50 mM, pH 10.2, containing 0.1 mM EDTA) and the reaction initiated with epinephrine (30 mM in 0.05% acetic acid). The rate of autoxidation

of epinephrine was measured at 480 nm for 180 s on a Hitachi U2000 spectrophotometer. SOD activity was expressed as units (U) SOD/mg protein, where 1 U of SOD is defined as the amount of enzyme present that inhibits the autoxidation of epinephrine by 50%.

Total glutathione (GSHt) levels

GSHt was measured by the enzymatic recycling procedure in which reduced glutathione (GSH) is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid, DTNB) to oxidized glutathione (GSSG), which is then reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GR back to GSH [33]. One hundred microliters of either tissue extract or known amounts of GSH standard was added to 800 µl of NADPH (0.3 mM) and 100 µl of DTNB (6 mM). The reaction was initiated with 10 µl of GR (50 U/ml). All solutions were made up in stock buffer (pH 7.5) containing sodium phosphate (125 mM) and sodium–EDTA (6.3 mM). The rate of DTNB reduction was measured at 412 nm continuously for 120 s. GSH was used as an external standard, and the level of GSHt in the samples was expressed as nmol GSH/g tissue.

Lipid oxidation (TBARS) assay

TBARS were measured by the method of Ohkawa et al. [34]. Tissue extract (100 µl) was added to a mixture containing 50 µl of sodium dodecyl sulfate (8.1%), 375 µl of acetic acid (20%, pH 3.5), and 375 µl of aqueous thiobarbituric acid (0.8%). The samples were heated in a boiling water bath for 60 min. The samples were allowed to cool at room temperature and then 1.25 ml of *n*-butanol:pyridine (15:1, v/v) was added. The mixture was shaken vigorously and centrifuged at 4,000 rpm (1,300 *g*) for 10 min. The upper colored organic layer was removed and read at 532 nm. The level of lipid peroxides in the samples was expressed as nmol malondialdehyde (MDA)/mg protein, using the molar extinction coefficient of MDA of 1.56×10^5 $(M\,cm)^{-1}$.

Protein oxidation (protein carbonyl) assay

Protein carbonyls are formed through oxidation of proteins by a variety of mechanisms. They are sensitive markers of oxidative injury. The customary way of analyzing protein carbonyls is a colorimetric procedure that measures binding of dinitrophenylhydrazine (DNPH). This test is an ELISA format using an anti-DNPH antibody developed by Buss et al. [35]. In this study we used the protein carbonyl enzyme (immunoassay) kit from Northwest Life Science Specialties (Biocell PC test, Cat. No. NWK-PCK-01). The quantity of protein carbonyls in each sample was determined by derivatizing with DNPH and measuring bound DNPH colorimetrically at 450 nm. The amount of protein carbonyl in each sample was calculated from a standard curve, using the protein carbonyl standards provided in the kit, and expressed as nmol protein carbonyls/mg protein.

Hexokinase (HK) activity

HK was measured according to the procedure of Knull et al. [36]. Tissue extract was added to the reaction mixture containing 100 µl each of glucose (33 mM), ATP (67 mM), $MgCl_2$ (67 mM), potassium Hepes (400 mM, pH 7.5), 1-thioglycerol (100 mM), $NADP^+$ (6.4 mM) and 10 µl of glucose 6-phosphate dehydrogenase (1 U) in a total volume of 1.0 ml. NADPH formation was followed at 340 nm for 3 min. HK activity was expressed as U of HK/g tissue, where 1 U of HK is defined as µmol NADPH formed/min using the molar extinction coefficient of NADPH, of 6.22×10^3 $(M\,cm)^{-1}$.

Statistical analysis

The values from duplicate samples for each biochemical measure (NO, SOD, GSHt, TBARS, protein carbonyls, and HK) were averaged to obtain 1 value point per brain region per animal. Four to six animals per biochemical measure were used. Each biochemical measure from the same brain region was analyzed on the same day. One-way ANOVA with post hoc Newman–Keuls was used to determine significant differences between SDSH, UCSH, and UCN rats for each biochemical measure for each brain region. Statistical significance was determined at the level of $p<0.05$.

Results

In this study we investigated whether (6 h) sleep deprivation under sustained hypoxia (SDSH) results in free radical production and/or oxidative stress in the rat neocortex, hippocampus, brainstem, and cerebellum. We did this by analyzing (i) NO levels, (ii) SOD activity, (iii) GSHt levels, (iv) TBARS, and (v) protein carbonyls, as well as (vi) HK activity.

Indicators of free radical production

Changes in NO levels in the neocortex, hippocampus, brainstem, and cerebellum of SDSH rats compared to UCSH and UCN rats are shown in Fig. 1. One-way ANOVA revealed statistically significant differences in NO levels between groups in the hippocampus ($F=4.57$, $p=0.03$). Significant increases in NO levels were observed between SDSH and UCSH rats (70%, $p=0.04$) as well as between SDSH and UCN animals (69%, $p=0.03$). On the other hand, no significant differences in NO levels were observed in the neocortex, the brainstem, or the cerebellum between rats sleep deprived under hypoxia and sleeping controls under either hypoxia or normoxia (SDSH vs either UCSH or UCN, $p>0.05$). Furthermore, 6 h of sustained hypoxia by itself did not significantly (UCN vs UCSH, $p>0.05$) change the level of NO in any of the brain regions studied here.

The level of oxygen free radicals was assayed in this study by measuring alterations in the activity of SOD and in the levels of GSHt. Changes in GSHt levels in various brain regions of SDSH rats compared to UCSH and UCN rats are shown in Fig. 2. One-way ANOVA revealed statistically significant differences between groups in the neocortex ($F=10.03$, $p=0.002$), the brainstem ($F=9.72$, $p=0.002$), and the cerebellum ($F=5.78$, $p=0.01$). Sleep deprivation under hypoxia significantly increased GSHt levels compared to sleeping controls under both hypoxia and normoxia, in the neocortex (SDSH vs UCSH, 35%, $p=0.003$, and SDSH vs UCN, 31%, $p=0.005$), the brainstem (SDSH vs UCSH, 21%, $p=0.009$, and SDSH vs UCN, 22%, $p=0.002$), and the

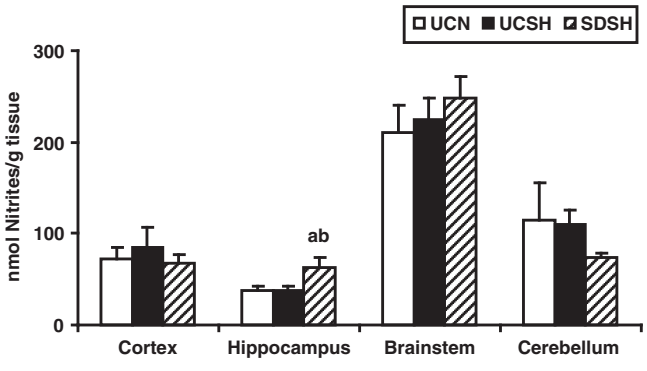

Fig. 1. Changes in NO levels in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM. [a]SDSH vs UCSH, $p<0.05$; [b]SDSH vs UCN, $p<0.05$.

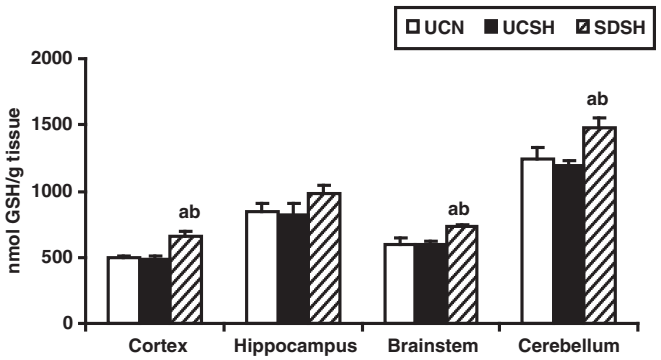

Fig. 2. Changes in total glutathione levels in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM. [a]SDSH vs UCSH, $p<0.05$; [b]SDSH vs UCN, $p<0.05$.

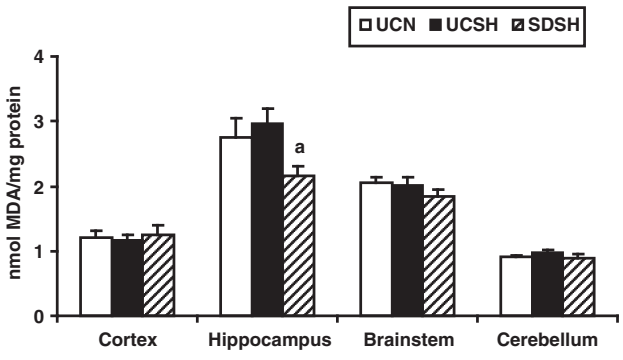

Fig. 4. Changes in levels of TBARS in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM. [a]SDSH vs UCSH, $p<0.05$.

cerebellum (SDSH vs UCSH, 24%, $p=0.01$, and SDSH vs UCN, 18%, $p=0.04$). On the other hand, sleep deprivation under hypoxia did not significantly alter SOD activity in any of the brain regions studied here, although SOD activity increased by 29% (SDSH vs UCSH, $p>0.05$) in the hippocampus and decreased by 26% (SDSH vs UCSH, $p>0.05$) in the cerebellum (Fig. 3) of sleep-deprived rats versus sleeping controls under hypoxia. A similar effect was observed in the hippocampus (increase) and cerebellum (decrease) with regard to NO levels. Sustained hypoxia alone did not produce any significant changes in either GSHt levels or SOD activity (UCSH vs UCN, $p>0.05$) in any of the brain regions studied here.

Markers of oxidative stress

Oxidative stress ensues when antioxidative mechanisms (such as SOD and GSHt) are unable to successfully scavenge the free radicals produced. In this study we measured oxidative stress by analyzing the levels of TBARS (as an indicator of oxidized lipids) and protein carbonyls (as an indicator of oxidized proteins). The levels of TBARS in the neocortex, hippocampus, brainstem, and cerebellum of SDSH rats compared to controls (UCSH and UCN) are shown in Fig. 4. Significant differences in the level of TBARS, between sleep deprived under hypoxia and sleeping controls, were observed only in the hippocampus (one-way ANOVA, $F=5.41$, $p=0.02$) where TBARS were significantly decreased by 27% in sleep-deprived hypoxic animals compared to hypoxic controls (SDSH vs UCSH, $p=0.02$) and insignificantly decreased by 21% compared to normoxic controls (SDSH vs UCN, $p=0.06$). The levels of protein carbonyls in the brain regions studied here are shown in Fig. 5. Large but nonsignificant decreases in protein carbonyl levels, between sleep deprived under hypoxia and sleeping control rats, were observed in the hippocampus (SDSH vs UCSH, −28%, and SDSH vs UCN, −23%, $p>0.05$). On the other hand, large but nonsignificant increases in protein carbonyls, between sleep deprived under hypoxia and sleeping controls, were observed in the brainstem (SDSH vs UCSH, 35%, and SDSH vs UCN, 23%, $p>0.05$). Sustained hypoxia by itself did not produce any significant changes in the levels of TBARS or protein carbonyls (UCSH vs UCN, $p>0.05$) in any of the brain regions studied here.

Source of free radicals

We hypothesize that glucose metabolism may be one potential source of free radicals. HK activity was used as a measure of alteration in glucose metabolism. Changes in HK activity in the neocortex, hippocampus, brainstem, and cerebellum of SDSH rats compared to UCSH and UCN rats are shown in Fig. 6. The neocortex was the only brain region studied here that showed a significant increase in HK activity with sleep deprivation under sustained hypoxia compared to hypoxic controls (SDSH vs UCSH, 34%, $p=0.03$). Sustained hypoxia alone did not increase the activity of HK in any of the brain regions studied here.

Discussion

This study is the first to demonstrate that sleep deprivation under sustained hypoxia increases antioxidant responses (NO production in

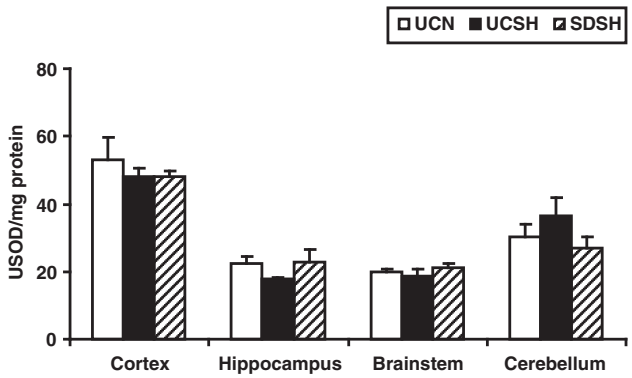

Fig. 3. Changes in SOD activity in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM.

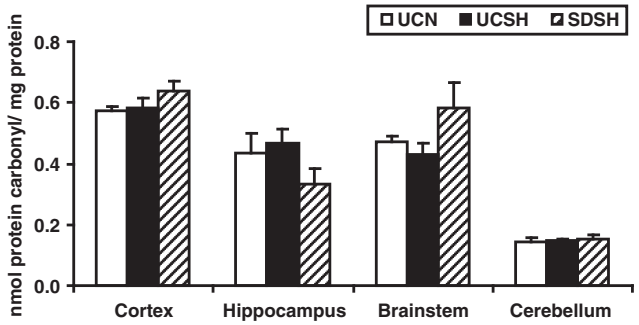

Fig. 5. Changes in levels of protein carbonyls in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM.

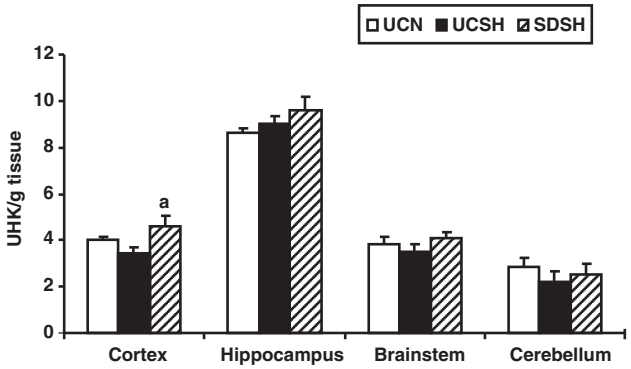

Fig. 6. Changes in HK activity in the neocortex, hippocampus, brainstem, and cerebellum of rats exposed to sleep deprivation under sustained hypoxia (SDSH) versus control animals left undisturbed under either sustained hypoxia (UCSH) or normoxia (UCN). Data are expressed as the mean ± SEM. [a]SDSH vs UCSH, $p<0.05$.

the hippocampus and GSHt levels in the neocortex, brainstem, and cerebellum) while decreasing lipid oxidation (TBARS) in the hippocampus, compared to both unhandled hypoxic and normoxic controls. Table 1 summarizes the findings of this study. We also showed increased HK activity in the neocortex of rats subjected to sleep deprivation under sustained hypoxia compared to sleeping controls under hypoxia, indicating that increased glucose metabolism may be one potential source of the elevated free radicals produced in this brain region.

We previously reported that under normoxia/room air, sleep deprivation increased GSHt levels and GPx activity in several rat brain regions, compared to control rats left undisturbed [23]. In this study we report that sleep deprivation under sustained hypoxia increased NO and GSHt levels, compared to sleeping controls under either hypoxia or normoxia. Taken together our findings suggest that 6 h of sleep deprivation (SD) under either normoxia [23] or hypoxia (this study) results in increased antioxidative responses, indicative of increased free radical production.

This study demonstrates increased NO levels in the hippocampus of rats subjected to sleep deprivation under sustained hypoxia, compared to sleeping controls under either sustained hypoxia or normoxia. NO is very unstable and rapidly oxidizes to its more stable metabolites, nitrates and nitrites. In this study, NO was measured by changes in total nitrite levels, after the reduction of nitrates to nitrites. NO is the only endogenous source of brain nitrates and nitrites, because dietary nitrates and nitrites do not cross the blood–brain barrier [37]. Increased NO levels, assessed by changes in total nitrite levels, have been observed in the perifornical lateral hypothalamus [38], the basal forebrain (BF), the frontal cortex (FC), but not the cingulate cortex [39], with SD. NO levels in the BF gradually increased during the first 3 h of SD, whereas NO levels in the FC increased only after 5 h of SD [39]. These authors also reported a similar time course of increase in inducible nitric oxide synthase mRNA and protein levels, mimicking the changes in NO levels in the BF and FC. On the other hand, Hsu et al. [40] reported that 5 days of total SD decreased NADPH diaphorase reactivity in the rat hippocampal CA1, CA2, and CA3 regions as well as in the dentate gyrus, suggesting a suppression of NO production in these regions. These authors also showed a concomitant decrease in neuronal nitric oxide synthase protein levels. Khadrawy et al. [41], on the other hand, showed that 72 h of REM sleep deprivation increased hippocampal NO production without affecting cortical NO production. The neuronal phenotype within the hippocampus, as well as the source of NO in our study, remains to be determined.

In our study we also showed that sleep deprivation under sustained hypoxia significantly increased GSHt levels in the rat neocortex, brainstem, and cerebellum compared to sleeping controls under either sustained hypoxia or normoxia. We previously reported that sleep deprivation under normoxia increased GSHt levels in the rat neocortex, brainstem, and basal forebrain and we speculated that this may account for the ability of sleep-deprived rats to compensate for any deficit in working memory [23]. D'Almeida et al. [24] reported that 96 h of REM sleep deprivation, on the other hand, significantly decreased GSHt levels in the rat hypothalamus. Glutathione is one of the most important physiological antioxidant involved in the detoxification of hydrogen peroxide and lipid hydroperoxide [42]. In the presence of transition metal ions (Cu^{2+} and Fe^{2+}) these peroxides can form the highly reactive hydroxyl radical, which can cause the oxidation of lipids, proteins, and nucleic acids, resulting in oxidative stress. Glutathione is found in the cytosol of cells in the range of 1–10 mM [43]. Glutathione exists in either its reduced (GSH) or its oxidized (GSSG) state. GSH is able to donate a reducing equivalent to other unstable molecules, such as reactive oxygen species, and in the process becomes GSSG. GSH can then be regenerated from GSSG by the enzyme glutathione reductase. In normal cells, more than 90% of the total glutathione pool is in the GSH form, with less than 10% in the GSSG form. However, even during oxidative stress, the ratio of GSH/GSSG remains very high [42], indicating that most of the glutathione pool exists as reduced glutathione, which is a very potent antioxidant.

In this study we also report that sleep deprivation under sustained hypoxia decreased the levels of TBARS in the rat hippocampus compared to sleeping controls under either sustained hypoxia or normoxia. On the other hand, Suer et al. [44] showed that 21 days of intermittent REM sleep deprivation (with 6 h of recovery sleep every day) increased lipid oxidation while decreasing SOD and GPx activities in the rat hippocampus. They further suggested that this alteration in the antioxidant defense system may account for the impaired maintenance of long-term potentiation observed in these rats. Khadrawy et al. [41] also reported that 72 h of REM sleep deprivation increased lipid oxidation in both the hippocampus and the

Table 1
Changes in different markers with normoxia, sustained hypoxia, and sleep deprivation under sustained hypoxia in various rat brain regions.

	Cortex	Hippocampus	Brainstem	Cerebellum
NO levels	NS	SDSH vs UCSH, $p<0.05$; SDSH vs UCN, $p<0.05$	NS	NS
GSHt levels	SDSH vs UCSH, $p<0.05$; SDSH vs UCN, $p<0.05$	NS	SDSH vs UCSH, $p<0.05$; SDSH vs UCN, $p<0.05$	SDSH vs UCSH, $p<0.05$; SDSH vs UCN, $p<0.05$
SOD activity	NS	NS	NS	NS
TBARS levels	NS	SDSH vs UCSH, $p<0.05$; SDSH vs UCN, $p=0.06$	NS	NS
Protein carbonyl levels	NS	NS	NS	NS
HK activity	SDSH vs UCSH, $p<0.05$	NS	NS	NS

GSHt, total glutathione; HK, hexokinase; NO, nitric oxide; NS, not significant; SDSH, sleep deprivation under sustained hypoxia; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; UCN, unhandled controls under normoxia; UCSH, unhandled controls under sustained hypoxia.

cortex. The levels of TBARS in the neocortex, brainstem, and cerebellum were not altered in our study. The increased GSHt levels in these brain regions suggest an increase in free radical production. The lack of change in the levels of TBARS indicates that the free radicals were successfully removed by the endogenous antioxidant (GSH), thus preventing oxidative stress.

We did not find any significant changes in the levels of carbonyl proteins in any of the brain regions studied here under any of the three treatment conditions. Carbonyl protein levels were decreased in the hippocampus and increased in the brainstem. These changes, although large, were insignificant because of the high degree of variation in the samples.

We speculated that one potential source of free radicals is elevated glucose metabolism. Reimund [2] hypothesized that free radicals accumulate during waking as a result of enhanced metabolic activity. In this study we showed that sleep-deprived rats under hypoxia had higher neocortical HK activity compared to control rats allowed to sleep under hypoxia. This increase in HK activity indicates enhanced glucose metabolism, which could result in elevated free radical production. We previously also reported that sleep deprivation under normoxia significantly increased HK activity in the rat neocortex and hypothalamus compared to sleeping controls [23]. The mitochondrial electron transport chain is a main source of free radicals and the mitochondrial enzyme Mn-SOD is a potent antioxidant [reviewed in 45,46]. We did not measure mitochondrial activity in our current study, but we will do so in future experiments.

Bailey et al. [47] reported increased cerebral oxidative and nitrosative stress in healthy men exposed to 9 h of hypoxia (12.9% oxygen) compared to normoxia. They showed increased arterial and venous levels of lipid hydroperoxides and alkoxyl-alkyl free radicals that correlated with increased acute mountain sickness/headache scores, as well as increased levels of 3-nitrotyrosine and decreased levels of plasma NO metabolites that correlated against acute mountain sickness/headache scores. Maiti et al. [48] reported that high altitude (hypobaric hypoxia) exposure increased the formation of reactive oxygen and nitrogen species in several rat brain regions. These authors showed that rats subjected to simulated altitude of 6100 m for 3 or 7 days exhibited decreased antioxidant responses and increased oxidative stress in the cortex, hippocampus, and striatum, compared to rats kept at sea level. These authors suggested that the hippocampus was the brain region most susceptible to the effects of hypoxia. We previously also reported that sustained/continuous hypoxia enhanced GR activity in the rat pons (after 6 h) and elevated TBARS levels in the rat cerebellum (after 1 day) [25], indicating that sustained hypoxia increases antioxidant responses and results in oxidative stress. In our current study, however, we did not observe any changes in the measured antioxidant responses (SOD activity and GSHt levels), nitrogen free radical production, or oxidative stress markers (TBARS and carbonyl proteins) due to hypoxia itself. This could be due to the short term exposure to sustained hypoxia, only 6 h. In future studies we will prolong the sleep deprivation procedure as well as include a sleep recovery group under sustained hypoxia, to see the effects on antioxidant responses and oxidative stress markers.

In conclusion, this study is the first to demonstrate that sleep loss under sustained hypoxia leads to increased nitric oxide production in the rat hippocampus and increased total glutathione levels in the rat neocortex, brainstem, and cerebellum, while decreasing hippocampal levels of TBARS. Hence, short-term insomnia under sustained hypoxia may serve as an adaptive response to prevent oxidative stress.

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