

Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem

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Received 5 May 2002; accepted 10 May 2002

Sleep deprivation by the disk-over-water technique results in a predictable syndrome of physiological changes in rats. It has been proposed that reactive oxygen species (ROS) may be responsible for some of these effects. A variety of antioxidative enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) help to regulate the level of ROS. In this study we investigated the effects of prolonged (5–11 days) sleep deprivation on the activities

of SOD and GPx as well as the metabolic activity of the mitochondria (using alamar blue) in several brain regions (cortex, hippocampus, hypothalamus, brainstem and cerebellum). We show that prolonged sleep deprivation significantly decreased Cu/Zn-SOD activity in the hippocampus and brainstem, suggesting an alteration in the metabolism of ROS resulting in oxidative stress. *NeuroReport* 13:1387–1390 © 2002 Lippincott Williams & Wilkins.

Key words: Brainstem; Glutathione peroxidase; Hippocampus; Oxidative stress; Reactive oxygen species; Sleep deprivation; Superoxide dismutase

INTRODUCTION

Rats subjected to prolonged sleep deprivation by the disk-over-water technique exhibit a predictable syndrome of physiological changes [1]. These include an initial increase followed by a decrease in body temperature below baseline, a loss in body weight despite increased food intake, suggesting increased energy expenditure and altered metabolism, as well as hyperkeratotic lesions on the paws and tail and ultimately death. The syndrome of physiological changes produced by sleep deprivation cannot be duplicated by any known behavioral or pharmacological manipulation. It has been proposed that reactive oxygen species (ROS), hypothesized to accumulate during waking as a result of enhanced metabolic activity, may be responsible for some of these effects [2].

Several studies have shown that there is greater metabolic activity during waking than during sleep [3]. Increased cerebral metabolism during waking results in increased oxidative metabolism, which enables greater energy production. The major energy production occurs in the mitochondria via oxidative phosphorylation through the electron transport chain (ETC). During the process of electron transfer through the ETC, a small percentage of electrons may leak, resulting in the formation of ROS (such as superoxide, $O_2^{\cdot-}$ and hydrogen peroxide, H_2O_2) and eventually to oxidative stress and cellular damage. Several enzymes expressed in the brain, including monoamine oxidase (MAO), tyrosine hydroxylase and L-amino oxidase, also produce H_2O_2 as a normal byproduct of their activity [4]. Calcium dependent activation of phospholipase A_2 releases arachidonic acid that yields $O_2^{\cdot-}$ through its subsequent metabolism by lipoxygenases [4].

Enhanced ROS production leads to oxidative stress and ultimately to cellular damage. Due to their extreme reactivity, it is difficult to detect and quantify ROS. Therefore, involvement of ROS in pathological conditions has generally been inferred from measurement of indirect markers of oxidative stress, such as changes in the activities of antioxidative enzymes. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are among the most important members of the antioxidant defense system. Alamar blue is used to examine the metabolic activity of the mitochondria. Alamar blue is a fluorescent dye that is an indicator of oxidation–reduction activity.

The aim of our study was to determine the effect of prolonged sleep deprivation on the activities of SOD and GPx as well as alamar blue in various rat brain regions. The disk-over-water method of sleep deprivation, designed by Rechtschaffen and colleagues [5], was used to achieve prolonged, well-controlled sleep deprivation. We show that prolonged sleep deprivation results in decreased Cu/Zn-SOD activity in the hippocampus and brainstem.

MATERIALS AND METHODS

Twelve male Sprague Dawley rats weighing 425–525 g at the time of surgery served as experimental subjects. Rats were purchased from Charles River and were housed in our animal facility at the V.A. Greater Los Angeles Healthcare System. All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Animals. All procedures were reviewed and approved by the Internal Animal Care and Use Committee of the

VAGLAHS and the Office for Protection of Research Subjects at UCLA.

The rats were prepared for chronic recording of sleep-wake variables by implanting electrodes for non-theta electroencephalogram (EEG), electromyogram (EMG) and theta EEG. To avoid inducing damage to the brain, electrodes were secured to the outside of the cranium, using a technique developed from our earlier study [6]. After surgery, rats were maintained in constant light (150 lux) to flatten their circadian rhythm. All rats underwent 10 days of post-operative recovery while in their cages. Both sleep deprived (SD) and yoked control (YC) rats received the same implants and were randomly assigned to experimental conditions. After recovery, pairs of rats were placed on the disk-over-water apparatus for 7 days of acclimatization. Apparatus temperature was regulated at 28–29°C by infrared lamps, which is thermoneutral for the rat. Food and water dispensers were suspended over the disk and were available *ad lib*. After the acclimatization period, 7 days of baseline followed, during which time the floor was removed and the rats were placed on the disk. The computer rotated the disk once every hour for 6 s in a random direction.

Amplified and filtered EEG, EMG and theta was continuously digitized and displayed on a computer monitor with the use of an integrated computer interface device (CED 1401 plus, supporting software, Spike 2; Cambridge Electronic Design 1401, London). EEG, EMG and theta data was stored on disk for subsequent analysis. During the deprivation, the disk was rotated in a randomly selected direction at 3.3 r.p.m. when sleep was detected in the SD rats. We have used the criteria developed by Bergmann *et al.* [5] for total sleep deprivation. The polygraph was run continuously, at 5 mm/s, recording EEG, EMG and theta from both SD and YC rats, during baseline (7 days) as well as during the deprivation period (5–11 days). This allowed visual scoring of sleep time and validation of the success of the deprivation and control procedures. Adjustments in thresholds were made once per day to assure that sleep was maximally deprived in SD rats and minimally disrupted in YC rats. Pairs of rats (YC and SD) were sacrificed at 10.00 a.m. Six sets of animals were subjected to the total sleep deprivation paradigm by the disk-over-water technique in this manner. All sleep state analyses were done manually from the paper polygraphic records. Sleep-wake states were defined on the basis of EEG and EMG patterns using standard criteria.

At the end of the experiment pairs of rats were decapitated. The brain was quickly taken out (2–3 min) and divided into 2 mm slices using a rat brain matrix. Different brain regions (1 × 2 mm area) were cut out using stereotaxic coordinates. The rostral to caudal extent we used for isolating brain regions were as follows: cortex (2 × lateral 5 mm area; somatosensory cortex, AP 1.20 to –5.80; weight = 0.2 g), hippocampus (1 × lateral 3 mm area; AP –3.80 to –5.80, weight = 0.11 g), hypothalamus (2 × 2 mm area; AP –0.26 to –2.12, weight = 0.05 g), brainstem (5 × 4 mm area; AP –8.80 to –10.80, weight = 0.13 g), and cerebellum (0.20 g). All the different brain regions were weighed immediately after dissection, frozen in liquid nitrogen and stored at –80°C. The different brain regions were homogenized in a hand held homogenizer with 12 strokes in cold homogenizing buffer (50 mM Tris-HCl, pH

7.5, 50 mM MgCl₂ and 20 mM EDTA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) to make a 20% homogenate (w/v). The homogenate was centrifuged in an Eppendorf micro-centrifuge (5415C) at 2000 rpm (320 × g) for 10 min at 4°C. The pellet was discarded and the supernatant re-centrifuged at 13 500 rpm (14 000 × g) for 30 min at 4°C. This supernatant was used for determining the activities of Cu/Zn-SOD and GPx. The pellet (containing mitochondrial proteins) was re-dissolved in the same homogenizing buffer, and used for determining the metabolic activity of the mitochondria (with alamar blue).

Prior to determining the enzyme activities, the protein content of the samples was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine plasma gamma globulin (Bio-Rad Laboratories, Richmond, CA) as the standard. The amount of protein in the standard and in the samples was determined on a microtitre plate reader at a wavelength of 750 nm.

Superoxide dismutase (SOD) activity was measured according to the method of Misra and Fridovich [7]. Tissue extract was added to 50 mM carbonate buffer (pH 10.2 containing 0.10 mM EDTA) and the reaction initiated with 30 mM epinephrine (Sigma, St. Louis, MO) in 0.05% acetic acid. The rate of autoxidation of epinephrine was measured at 480 nm for 180 s on a Hitachi U2000 spectrophotometer. Superoxide dismutase activity was expressed as units (U) of SOD/mg of protein, where one unit of SOD is defined as the amount of enzyme present that inhibits the autoxidation of epinephrine by 50%. Superoxide dismutase activity from SD rats was compared to YC rats.

Glutathione peroxidase (GPx) activity was determined according to the method of Somani and Husain [8]. The reaction mixture consisted of 50 mM phosphate buffer, 100 µl of 0.01 M GSH (Sigma, St. Louis, MO), 100 µl of 1.5 mM NADPH (Sigma, St. Louis, MO) and 100 µl of 0.24 units GR (Sigma, St. Louis, MO). Tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Then, 100 µl of 12 mM t-butyl hydroperoxide (Sigma, St. Louis, MO) was added to 90 µl of the tissue reaction mixture and the rate of oxidation of NADPH was measured at 340 nm for 180 s. The molar extinction coefficient of 6.22×10^3 (Mcm)⁻¹ was used to determine GPx activity. Glutathione peroxidase activity was expressed as mM NADPH oxidized/min/mg protein. Glutathione peroxidase activity from SD rats was compared to YC rats.

Alamar blue was used to examine the metabolic activity of the mitochondria in synaptosomes made from mitochondrial extracts [9]. Alamar blue is a fluorescent dye that is an indicator of oxidation-reduction activity. Synaptosomes were incubated at 37°C in a buffer containing 10% (final volume) alamar blue (Trek Diagnostic Systems, Inc. West Lake, Ohio). Synaptosomes were centrifuged at 5000 rpm (2000 × g) for 5 min and the supernatant was transferred to a 96 well plate. Fluorescence was quantified using a Millipore Cytofluor 2300 fluorescence plate reader (530 nm excitation and 590 nm emission). Mitochondrial metabolic activity, assessed with alamar blue, was expressed as fluorescence intensity/mg protein. Mitochondrial metabolic activity from SD rats was compared to YC rats.

The values from triplicate samples for each biochemical measure were averaged to obtain one value point per

animal. Comparisons of SOD, GPx and alamar blue activity in different brain regions was done using analysis of variance. Significant differences between paired sets were determined by *post-hoc* Bonferroni analyses.

RESULTS

After prolonged (5–11 days) sleep deprivation pairs of rats (SD and YC) were sacrificed. Since it is difficult to precisely control the amount of sleep loss in SD and YC rats from one set to another, all analyses were made between SD and YC rats within the same set. In every set the SD rat lost more sleep than the YC rat. The rats were sacrificed when death seemed imminent regardless of the amount of sleep loss. The rats were killed at 10.00 a.m. by decapitation on the day of sacrifice. Both animals were killed within 30 min of each other.

SD rats lost $72 \pm 7\%$ (range 56–85%) of their total sleep, while YC rats lost $39 \pm 5\%$ (range 6–57%) of their total sleep. The amount of sleep loss was calculated as:

$$\frac{(\text{average baseline sleep time} - \text{average sleep time during the deprivation period})}{\text{average baseline sleep time}}$$

$\times 100\%$

SD rats lost 74.0 ± 23.1 h of accumulated sleep (corresponding to 9.4 ± 3.0 h/day) while YC rats lost 43.2 ± 19.8 h of accumulated sleep (corresponding to 5.9 ± 2.2 h/day). The SD rats not only had a reduction in total sleep time, but the NREM sleep they did get had a shorter mean bout duration. The mean bout duration of NREM sleep was 70 ± 9 s in SD rats and 186 ± 39 s in YC rats. The number of NREM sleep bouts was not significantly different between SD and YC rats ($t=0.75$, $df=4$, $p=0.5$). The mean bout duration of REM sleep was not significantly different between SD (124 ± 13 s) and YC (124 ± 51 s) rats. However, SD rats had significantly fewer REM sleep bouts compared to YC rats ($t=2.99$, $df=4$, $p=0.04$).

Biochemical assays were performed on the cortex, hippocampus, hypothalamus, brainstem and cerebellum to determine if prolonged sleep deprivation resulted in changes in the activities of any of the oxidative stress markers studied. The results are shown in Table 1.

Copper-zinc superoxide dismutase (Cu/Zn-SOD), the cytosolic isoform of SOD, is responsible for scavenging superoxide ions ($O_2^{\cdot-}$), which is the major reactive oxygen species (ROS) in the cell. Separate one-way ANOVAs for each brain region revealed significant differences in SOD activity in the hippocampus and brainstem ($F(1,58)=4.467$; $p < 0.04$). Pair-wise *post hoc* comparisons indicated that SOD

activity was significantly reduced in SD compared to YC rats both in the hippocampus (-10% , $t=3.70$, $df=5$, $p=0.01$) as well as in the brainstem (-21% , $t=3.00$, $df=5$, $p=0.03$).

Glutathione peroxidase (GPx) is responsible for scavenging hydrogen peroxide (H_2O_2) produced by the reaction of $O_2^{\cdot-}$ with SOD. No significant change in the activity of GPx was observed between SD and YC rats in any of the brain regions studied (Table 1).

Mitochondrial metabolic activity was assessed by using alamar blue, a dye whose fluorescence intensity depends on mitochondrial function. No significant difference in fluorescence intensity between SD and YC rats was found in any of the brain regions studied (Table 1).

DISCUSSION

Our results indicate that prolonged sleep deprivation by the disk-over-water technique leads to a significant decrease in the activity of Cu/Zn-SOD in the hippocampus and brainstem. No changes in the activities of Cu/Zn-SOD or GPx nor in alamar blue intensity were found in the cortex, hypothalamus or cerebellum, suggesting that the brain is not uniformly vulnerable to the effects of sleep deprivation. We speculate that certain neuronal populations may be more vulnerable to oxidative stress as a result of a greater oxidative burden, or, alternatively, lower antioxidant protection. It is likely that sleep deprivation causes oxidative stress by preventing non-REM sleep related reduction in the tiring of most neuronal groups, and thus the accompanying reduction of brain metabolism [3,10].

A steady state level of $O_2^{\cdot-}$ and H_2O_2 is always present in cells due to normal metabolism. SOD and GPx are responsible for the degradation of $O_2^{\cdot-}$ and H_2O_2 respectively. Therefore, changes in the activities of SOD and GPx, and also in alamar blue would reflect changes in the levels of ROS (such as $O_2^{\cdot-}$ and H_2O_2). The decreased activity of Cu/Zn-SOD in our experiments could be the result of increased protein inactivation or degradation or decreased gene expression, due to an increase in the level of ROS production. It has been shown that under oxidative stress conditions, the overproduction of ROS is able to inhibit SOD activity [11]. This would create a positive feedback loop resulting in further ROS accumulation. ROS can cause structural changes to proteins resulting in spontaneous fragmentation and/or increased proteolytic susceptibility [12]. Davies and co-workers [12] have shown that SOD exposed to hydroxyl radicals ($\cdot OH$) is an excellent proteolytic substrate. Conversely, the level of ROS produc-

Table 1. Regional brain Cu/Zn-SOD, GPx and mitochondrial metabolic activity after sleep deprivation.

Brain region	Cu/Zn-SOD activity (U SOD/mg protein)		GPx activity (mM NADPH oxidized/min/mg protein)		Mitochondrial metabolic activity (fluorescence/mg protein)	
	YC	SD	YC	SD	YC	SD
Cortex	38.0 ± 11.2	33.2 ± 9.7	0.06 ± 0.01	0.07 ± 0.01	$52\,298 \pm 8913$	$59\,782 \pm 14\,895$
Hippocampus	33.1 ± 4.1	296 ± 3.6^a	0.04 ± 0.01	0.05 ± 0.01	$56\,394 \pm 7417$	$57\,400 \pm 13\,523$
Hypothalamus	14.2 ± 4.3	13.3 ± 3.4	0.08 ± 0.01	0.08 ± 0.01	NA	NA
Brainstem (pons and medulla)	890 ± 24.3	71.8 ± 20.5^a	0.10 ± 0.01	0.11 ± 0.01	$15\,965 \pm 2853$	$14\,689 \pm 3079$
Cerebellum	54.8 ± 22.4	63.8 ± 26.9	0.06 ± 0.01	0.07 ± 0.01	$73\,405 \pm 2227$	$81\,295 \pm 3191$

Values are expressed as mean \pm s.e.m. YC=yoke control; SD=sleep deprived.

^aStatistically different from yoke control ($p < 0.05$). NA = data not available.

tion may not have been altered with sleep deprivation, but the decreased SOD activity would fail to detoxify $O_2^{\cdot -}$ resulting in oxidative stress nonetheless. The lack of effect on GPx and alamar blue suggest that ROS production may not have been altered by prolonged sleep deprivation.

Decreased SOD activity has been observed under various conditions linked with oxidative stress, including kainic acid [13], electroconvulsive shock (ECS)-induced seizures [14], pilocarpine-induced epilepsy [15], subarachnoid-induced hemorrhage [16], 6-hydroxydopamine (6-OHDA) [17], in aged rats [18] and in Alzheimer's brains [19,20].

We have previously reported that prolonged sleep deprivation by the disk-over-water technique resulted in increased amino cupric silver staining in the supraoptic nucleus (SON) of the hypothalamus [6]. Amino cupric silver staining is thought to result from the precipitation of silver around chemical reducing groups present in damaged subcellular structures [21]. Oxidative stress resulting in enhanced ROS production has been shown to damage proteins, lipids and DNA. The increase in amino cupric silver staining in the SON of sleep deprived rats may therefore arise from oxidative stress induced damage. Although no changes in Cu/Zn-SOD, GPx or mitochondrial metabolic activity (by alamar blue) was observed in the hypothalamus, we cannot rule out the possibility that oxidative stress is involved, since other markers not assayed here may be responsible for the amino cupric silver staining. Also, the SON constitutes only a very small area of the hypothalamus and biochemical assays performed on the hypothalamic homogenate may not be sensitive to such localized changes. D'Almeida and co-workers [22] reported a significant decrease in total glutathione (GSHt) levels in the hypothalamus of REM sleep deprived (for 96 h) rats. Reductions in glutathione levels are commonly used as a measure of oxidative stress [23].

CONCLUSION

Our current study shows that prolonged sleep deprivation results in a significant decrease in the activity of Cu/Zn-

SOD in the rat hippocampus and brainstem. This decrease may be due to the degradation of antioxidative enzymes, after prolonged activation in waking, resulting in increased ROS production, or, alternatively, may be due to increased ROS production. Ultimately, this would lead to oxidative stress resulting in damage to brain structures. To the best of our knowledge, this is the first study to link oxidative stress with prolonged sleep deprivation.

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Acknowledgements: This research was supported by the Medical Research Service of the Department of Veterans Affairs and HL 41370 and MH 64109.