Antioxidant defense responses to sleep loss and sleep recovery

Carol A. Everson, Christa D. Laatsch, and Neil Hogg. Antioxidant defense responses to sleep loss and sleep recovery. Am J Physiol Regul Integr Comp Physiol 288: R374–R383, 2005. First published October 7, 2004; doi:10.1152/ajpregu.00565.2004.—Sleep deprivation in humans is widely believed to impair health, and sleep is thought to have powerful restorative properties. The specific physical and biochemical factors and processes mediating these outcomes, however, are poorly elucidated. Sleep deprivation in the animal model produces a condition that eventually becomes highly lethal, lacks specific localization, and is reversible with sleep, implying mediation by a biochemical abnormality. Metabolic and immunological consequences of sleep deprivation point to a high potential for antioxidant imbalance. The objective, therefore, was to study glutathione content in the liver, heart, and lung, because glutathione is considered a major free radical scavenger that reflects the degree to which a tissue has been oxidatively challenged. We also investigated major enzymatic antioxidants, including catalase and glutathione peroxidase, as well as indexes of glutathione recycling. Catalase activity and glutathione content, which normally are tightly regulated, were both decreased in liver by 23–36% by 5 and 10 days of sleep deprivation. Such levels are associated with impaired health in other animal models of oxidative stress-associated disease. The decreases were accompanied by markers of generalized cell injury and absence of responses by the other enzymatic antioxidants under study. Enzymatic activities in the heart indicated an increased rate of oxidative pentose phosphate pathway activity during sleep deprivation. Recovery sleep normalized antioxidant content in liver and enhanced enzymatic antioxidant activities in both the liver and the heart. The present results link uncompensated oxidative stress to health effects induced by sleep deprivation and provide evidence that restoration of antioxidant balance is a property of recovery sleep.

Sleep deprivation; sleep rebound; oxidative stress; glutathione recycling; resistance to disease

SLEEP DEPRIVATION IS CONSIDERED a risk factor for disease (45b). The physical and biochemical changes produced by sleep and sleep deprivation that result in health consequences, however, are largely unknown. A unique clinical profile has emerged from comparative studies in laboratory animals. The principal signs include abnormally high rates of food ingestion without weight gain (16, 22), early and sustained reductions in anabolic hormones (17, 19), and early transient infections of internal tissues by opportunistic microorganisms (21), which, after 3 wk, lead to advanced morbidity (16, 49) and lethal septicemia (15). Despite many investigative attempts, sleep deprivation effects have not been localized to a specific tissue or system, and structural damage has not been found by light microscopy of major peripheral organs (15, 25). Structural damage in the brain has been found only in the supraoptic nucleus of the hypothalamus in rats sleep deprived for 8–10 days, and its clinical significance still is speculative (14). Biomarkers that identify the sleep-deprived state and might be used to assess the physiological impact of sleep deprivation have remained undiscovered. Corticosteroids and cortisol, the oft-presumed principal mediators of metabolic and immune-related abnormalities, remain unchanged or decreased in sleep-deprived rats (17, 19) and humans (37, 38, 64) in studies that controlled for extraneous variables that may elicit behavioral distress. Recovery sleep after sleep deprivation in the animal model reverses all known signs (such as hypercatabolism and thyroid hormone deficiency), often even during advanced morbidity (18). These events, among other evidence, indicate that the consequences of sleep deprivation, although highly lethal, are also highly reversible. Because morbidity develops in sleep-deprived rats without apparent structural damage, and in a manner reversible with sleep, the state may be considered toxicogenic.

The present study investigated oxidative stress as a biological intermediary to the health effects imposed by sleep deprivation. Oxidative metabolism and energy production in the body generate free radicals and nonradical derivatives of oxygen and of nitrogen (reviewed in Ref. 30). Although these products are involved in normal cell regulation and signal transduction, an imbalance between their generation and the antioxidant defense system results in oxidative stress. The hypermetabolism and immunopathology in sleep-deprived animals (2, 21) are expected to produce excessive metabolic and oxidative burdens. Oxidative stress, in turn, may perpetuate metabolic dysfunction (62), immune compromise (63, 68), and other health impairments.

Little is known regarding whether oxidative stress is an important consequence of sleep deprivation. Reimund (50) theorized in 1994 that sleep increases the efficiency of antioxidant mechanisms in the brain. Tufik and colleagues reported that 96 h of deprivation of paradoxical sleep [PS; also known as rapid eye movement (REM) sleep] produced heterogeneous alterations of various antioxidant indexes in rat brain (10, 11), although confounds in the live-animal experimental design also have been cited (9). Ramanathan et al. (48) reported a significant decrease in superoxide dismutase (SOD) activity in the hippocampus and brain stem, but not in the cerebral cortex, hypothalamus, or cerebellum in rats sleep deprived for 5–11 days. They also did not find changes in regional brain glutathione peroxidase (GPX) (an enzymatic antioxidant), or in Alamar blue staining of synaptosomes in an assessment of the activity of mitochondria (48). Gopalakrishnan et al. (28) reported likewise that SOD activity was unchanged in cerebral cortex. They also did not detect changes in liver or muscle

Address for reprint requests and other correspondence: C. A. Everson, Medical College of Wisconsin, VAMC, Neurology Research 151, 5000 West National Ave., Milwaukee WI 53295 (E-mail: ceverson@mwc.edu).

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tissue for the parameters: SOD activity, thiobarbituric acid-reactive substances (considered a nonspecific end product of lipid peroxidation), carbonyls (an index of protein oxidation), or oxidation of dichlorofluorescein in animals sleep deprived for 0–8 h or 3–14 days (28). Some potential caveats bear on generalizing their findings, however, such as their use of dichlorofluorescein in freezer-preserved tissue rather than in living cells. Studies of oxidative stress in clinical sleep research mainly have focused on obstructive sleep apnea, a disorder marked by repeated cessation of breathing and profoundly disrupted sleep. Oxidative stress in sleep apnea is thought to be produced by hypoxic events and by hypoxia-reperfusion injury, and in this way it contributes to cardiovascular complications and inflammatory processes (e.g., Refs. 4, 39, 55). A role for disrupted sleep itself in the metabolic complications of sleep apnea has been implied by some of the evidence but not fully explored (60). Accordingly, the meager collective evidence thus far has not clarified whether clinically meaningful oxidative stress is induced during sleep deprivation.

The focus of the present studies is on the antioxidant status of peripheral tissues, rather than the brain, during sleep deprivation and during subsequent sleep. This is because the principal signs of sleep deprivation in the animal model are systemic. Additionally, most comorbidities linked to disturbed or impaired sleep, such as cardiovascular disease, diabetes, and arthritis (45a, 45b), are described foremost by their systemic manifestations. Animals were studied for 5 and 10 days of sleep deprivation to increase the likelihood that factors are operating that might be reflected in measurable changes in tissues but short enough to preclude the effects associated with advanced morbidity that develop after a mean of 20 days (15, 22). As with experimental deprivation of other basic requirements, such as food and water, the deprivation of sleep must be prolonged and fairly constant to allow signs to emerge that permit study and tracing of their evolution. For these reasons, our animal model is not considered a replication of typical real-life human circumstances. Nonetheless, it is worth pointing out that 5–10 days of profound sleep deprivation are not unheard of in humans. It is common in the critically ill, who are near total loss of sleep of slow-wave sleep and/or REM sleep for periods of 5–14 days (46). The present study included a postdeprivation period of ad libitum sleep following profound sleep deprivation. The duration of this period was 48 h and chosen because the rebound of REM sleep (also known as PS) is most intense during the first 24–48 h (18, 23).

We focused on the principal marker for antioxidant depletion or repair, glutathione, and the enzymatic antioxidants associated with the glutathione recycling pathway. GPX reduces hydrogen peroxide (H₂O₂) and other hydroperoxides, such as lipid hydroperoxides, in a reaction coupled to the oxidation of reduced glutathione (GSH). This in turn creates glutathione disulfide (GSSG) and H₂O. Glutathione reductase (GSSG-R) catalyzes the reaction of reducing GSSG back to GSH, which is the reduced form that can be used for antioxidant activities. The cofactor for GSSG reduction is NADPH, provided by means of the oxidative pentose phosphate pathway. In this pathway, key enzyme activity indicators of the rate of NADPH regeneration from available NADP are glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD). Besides glutathione content and these related recycling enzymes, we determined catalase activity, which is an antioxidant defense enzyme that catalyzes the conversion of H₂O₂ to water and O₂. Catalase, located in the peroxisome, is the counterpart to GPX, which is mainly located in the cytosol. The tissues of interest were the liver, because it is the main detoxifying tissue of the body, and the heart and the lung, because of potential cardiovascular and pulmonary implications.

Oxidative stress may lead to cell injury and, if not repairable, to cell death by apoptosis or by necrosis. Many investigators linking oxidative stress to cell injury do so by measuring aminotransferases in plasma (e.g., Refs. 47, 57, 61). The appearance of increased plasma aminotransferases indicates cell membrane damage and leakage from the cytoplasm. We report here on the results of these parameters obtained from a subgroup of animals catheterized for blood drawing under freely moving conditions.

The principal findings of the present investigation were marked decreases in both liver glutathione and catalase activity in sleep-deprived animals, without detectable increases in recycling activities, suggesting uncompensated oxidative stress. In the heart, at the rate at which the pentose phosphate pathway was operating, which, in turn, determines the NADPH supply for glutathione reduction, appeared to be accelerated during sleep deprivation. These conditions were coincident with marked increases in serum aminotransferase concentrations, indicative of cell damage. Recovery sleep was associated with restoration or accentuation of antioxidant activities and antioxidant capacity in both the liver and the heart.

METHODS

Animals and Surgical Procedures

Animal care and use procedures were carried out in accordance with the National Institutes of Health guidelines on the care and use of animals and an approved animal study protocol at the Medical College of Wisconsin and the Zablocki Veterans Affairs Medical Center.

Surgical anesthesia and analgesia were produced by ketamine HCl (100 mg/kg ip), xylazine HCl (2.4 mg/kg im), and atropine sulfate (0.1 mg/kg im), and supplementary doses of ketamine HCl (10 mg/kg ip) were administered as needed. All subjects were implanted with cortical and muscle electrodes to record wakefulness and specific sleep stages, as described previously (3). All surgical procedures were performed during a single episode of anesthesia to avoid confounding effects of anesthesia and surgery during the sleep deprivation period.

Animals in Which Antioxidant Parameters Were Determined

For measurement of antioxidant parameters in the liver, the heart, and the lung, 26 experiments were completed to study 52 animals. Each experiment consisted of procedures conducted on two animals at a time: either a sleep-deprived animal and an animal yoked to the experimental conditions, or two operated control animals housed under baseline conditions. There were six to eight animals in each subgroup of animals catheterized for blood drawing under ad libitum sleep conditions, 5 or 10 days of sleep deprivation and yoked conditions, and 2 days of recovery sleep after 10 days of sleep deprivation and yoked conditions. Male Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN) and weighed 457 (SD 35) g and were 21.7 (SD 1.3) wk old at the time of surgery. After completion of each duration under study, the animals were gently and deeply anesthetized by halothane inhalation followed by ketamine HCl (50 mg/kg ip), xylazine HCl (2.4 mg/kg im), and atropine sulfate (0.1 mg/kg im), and exsanguinated by
cardiac puncture. After exsanguination of each animal, the liver, lung, and heart tissues were quickly dissected, rinsed in sterile saline, blotted, parceled, and processed for assay or frozen in liquid nitrogen for batch assay. Necropsy procedures were begun at the same time of day.

**Animals and Procedure for Sampling Blood for Measurement of Plasma Aspartate Aminotransferase, Alanine Aminotransferase, and γ-Glutamyltransferase**

Four experiments were conducted in which blood was sampled through indwelling catheters under the animals’ freely moving conditions during baseline and during up to 19 days of the experimental period in four sleep-deprived animals and four yoked animals. For these animals, measurements of food intake, body weight, hematologic variables, and clinical chemistry relevant to hypermetabolism and nutritional status have been reported (22). Measurements of plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) previously were not analyzed, and they are reported here because of their strong application as markers of cell injury. There was not a sleep recovery condition for these catheterized animals.

These eight animals also were male Sprague-Dawley rats (Harlan, Frederick, MD) that weighed 391 (SD 19) g at the time of surgery and were 18.0 (SD 2.6) wk old. The indwelling catheters were implanted according to procedures described elsewhere (3, 19, 22). Briefly, during the same surgery in which recording electrodes were implanted, a venous catheter was inserted in the external jugular and advanced to the right atrium. The catheter exited at the back of the head, where it was anchored to an electrode head plug assembly and extended up along a 45-cm EEG recording cable for blood sampling without handling the animal. Catheters were flushed daily with heparinized saline containing a small amount of ampicillin (1.25% wt/vol). Beginning 4 days after surgery, 75 units of streptokinase in 150 μl of heparinized saline were infused (1 ml/min) to fill the catheter lumen to reduce the occurrence of fibrin clot blockages. Blood samples were collected at the same time of day according to established methods (3) and included volume and red blood cell replacement.

**Animal Environmental Conditions**

Rats were kept under conditions of constant light to diminish the influence of the amplitude of the circadian rhythm on factors under study. Ambient temperature was maintained within the thermoneutral zone for rats at 28°C (59) by thermostatically controlled heat lamps. Food and water were available ad libitum. During and after the baseline period, the rats were fed a balanced, purified diet that was isoenergetic to a normal diet and augmented with protein, as used previously (22). All animals were allowed to recover from surgery for ≥7 days before the start of baseline conditions.

**Procedure for Producing Sleep-deprived, Yoked, and Baseline Control Animals**

The experimental apparatus and procedures used in the present experiments were developed by Bergmann et al. (3) with subsequent minor modifications. The method, which has been validated for its selectivity of sleep deprivation (2, 3, 16, 22, 49), features a benign arousal stimulus, freedom of movement, and comparison conditions of partial or full sleep opportunities, described in more detail and illustrated elsewhere (3, 22). Only one pair of animals was studied at a time, consisting of a rat to be sleep deprived and another rat yoked to the same experimental conditions. The two animals are housed inside a large Plexiglas enclosure, one on each side of a large platform (46-cm diameter) divided into two sides. Beneath the platform, and extending to the cage perimeter, is shallow water that serves as a soft boundary. The long recording cable, fastened to the electrode head plug assembly on each rat, is connected to a 360° commutator and counterclocked boom assembly that provide free movement within each side of the enclosure. The rats almost always avoid the water and stay on the platform where they can eat, sleep, and explore normally. Seven days are allotted under baseline conditions, during which sleep occurs an average of 54% of the time. Of total time, 48% is non-REM (NREM) sleep (comparable to human stages I–IV), 6% is PS, and 46% is wakefulness (15, 16, 20, 22). The platform is rotated once per hour for 6 s to acquaint the rats with platform movement.

During the sleep deprivation phase, sleep onset in the rat to be sleep deprived is detected by digitized amplitude changes in cortical EEG, cortical theta, and electromyographic variables (collectively, the EEG) by a microprocessor programmed by visual analysis of the behavior and the analog EEG recording. Detection of sleep onset produces a rotation of the housing platform for 6 s. There is no platform rotation when the sleep-deprived rat is engaged in behaviors other than trying to sleep. The method produces a consistent amount of sleep reduction in our studies to <10% of total time. The sleep obtained is composed mostly of transitional sleep and fragmented high-amplitude NREM sleep. PS comprises <1% of total time (15, 16, 20, 22). The percentage of time the platform is rotated is 18–22% and does not increase across experimental days (16, 22).

The paired comparison animal is considered yoked to the conditions of the sleep-deprived animal. The yoked animal may sleep when the sleep-deprived rat is engaged in behaviors other than trying to sleep, because the platform is stationary. Each paired yoked animal is frequently awakened if asleep because of the shared platform rotation. The sleep of yoked rats, therefore, is highly fragmented and reduced in total amount to 38% in NREM sleep and 3% in PS (15, 16, 20, 22). Yoked rats typically exhibit several signs in the same direction as do sleep-deprived rats but not to the same degree (e.g., Ref. 16), reflecting interrupted sleep processes.

**Assay of Antioxidant Parameters**

**Glutathione.** Measurements of total glutathione content were made first in fresh and later in frozen tissues, because differences in results due to immediate assay vs. preservation by fast freezing were not detected in control experiments. Tissues were homogenized 1:5 in 5% S-sulfocysteine acid containing 1 mM diethylenetriaminepentaacetic acid and then centrifuged at 5,000 g for 10 min at 4°C. Aliquots of supernatant were diluted 1:5 for the liver and 2:5 for each heart and lung in the same homogenization solution. Glutathione content was determined by the GSSG-R recycling assay, a kinetic method of absorbance spectrophotometry developed by Griffith (29). An aliquot of specimen was added to the reaction mixture that contained 103 mM potassium phosphate buffer (pH 7.5) containing EDTA (5.17 mM), β-NADPH (0.215 mM), and 5,5′-dithiobis(2-nitrobenzoic acid) (0.513 mM). GSSG-R (185 U/ml) was added, and the absorbance at 412 nm was recorded every 2.5 s during 60 s. The initial rate of the reaction from 5 to 60 s was calculated. Glutathione concentration was derived from a standard curve of l-glutathione (reduced form).

**Catalase activity.** Tissues were homogenized 1:10 in potassium phosphate buffer (50 mM, pH 7.4) and centrifuged at 3,000 g for 10 min at 4°C. The assay of catalase activity was based on the methods reported by Aebi (1), with modifications by Karthikeyan et al. (34) that included centrifugation at 3,000 g to remove nucleic acids and membrane components. The supernatant, further diluted 1:40 for liver, 1:50 for heart, and 1:100 for lung, was mixed with potassium phosphate buffer in a cuvette and incubated for 2 min at 25°C. The reaction was started by the addition of H₂O₂ (30 mM). Absorbance was measured at 240 nm each second during 35 s. The initial rate of reaction from 0 to 30 s was calculated. Catalase concentration was derived from a standard curve prepared by using catalase from bovine liver (Sigma). One unit decomposes 1 μmol H₂O₂ per minute at pH 7.0 and 25°C.

**G-6-PD and 6-PGD.** Tissues were homogenized 1:20 in 250 mM sucrose and then centrifuged at 9,000 g for 20 min at 4°C, according to the method of Dessi et al. (13). Measurement of G-6-PD and...
6-PGD was performed according to Glock and McLean (26), except that we used Tris-hydrochloride (Tris-HCl) instead of glycyglycine, which is an established modification of the procedure. Volumes were adjusted for the size of the specimen aliquot. For measurement of combined G-6-PD and 6-PGD, Tris-HCl (250 mM, pH 7.6) was combined with 100 mM magnesium chloride, β-NADP (0.207 mM), glucose-6-phosphate (G-6P; 50 mM disodium salt), and 6-phosphogluconic acid (6-PG; 50 mM trisodium salt). The reagent mixture was incubated at 37°C for 2 min before the addition of tissue supernatant. The absorbance at 340 nm was recorded every 15 s during 5 min. The initial rate of the reaction was calculated over the linear portion of the curve. Combined G-6-PD and 6-PGD concentrations were determined from a standard curve prepared using G-6-PD (from baker’s yeast, Sigma) and 6-PGD (from Saccharomyces cerevisiae, Sigma). Measurement of 6-PGD alone was accomplished by the same procedures, except that G-6P was excluded from the reaction mixture. One unit of G-6-PD oxidizes 1 μmol of G-6P to 6-PG per minute in the presence of NADP at pH 7.4 and 25°C. One unit of 6-PGD oxidizes 1 μmol of 6-PG to ribulose-5-phosphate per minute in the presence of NADP at pH 7.4 and 37°C.

GSSG-R. Tissues were homogenized 1:10 in potassium phosphate buffer (75 mM, pH 7.6), containing 2 mM dithiothreitol and 300 mM sucrose (31), and centrifuged at 15,000 g for 20 min at 4°C. The assay for GSSG-R activity was based on the methods of fantomasi et al. (31) and Goldberg and Spooner (27), with modifications for sample size and use of a standard curve. The supernatant was diluted in the homogenization buffer for the liver (1:4) and for the lung (1:2). The sample then was mixed with potassium-phosphate buffer (120 mM, pH 7.2), disodium EDTA (15 mM), distilled water, and GSSG (65.5 mM), and then incubated 2 min at 37°C. The reaction was started by the addition of β-NADPH (8.4 mM), and the absorbance at 340 nm was measured every 15 s during 5 min. The rate of the reaction from 2 to 4 min was calculated. GSSG-R activity was derived from a standard curve prepared by using GSSG-R (from baker’s yeast, Sigma). One unit of GSSG-R degrades 1 μmol GSSG per minute at pH 7.6 and 25°C.

GPX activity. Tissues were homogenized 1:10 in potassium phosphate buffer (75 mM, pH 7.0) containing 2 mM dithiothreitol and 300 mM sucrose, to help prevent oxidation of the sample during preparation (31, 44), and centrifuged at 20,000 g for 3 min at 4°C. The GPX assay was based on methodology described by Khaper and Singal (35). The sample supernatant was further diluted in potassium phosphate buffer (liver 1:40, heart 1:10, lung 1:4) before addition of the sample to the reaction buffer. The reaction buffer consisted of potassium phosphate buffer (75 mM, pH 7.0) containing sodium azide (2.8 mM), disodium EDTA (0.7 mM), GSH (1.25 mM), GSSG-R (1.25 U/ml), and β-NADPH (0.125 mM). The mixture was incubated 2 min at 37°C. The reaction was started by the addition of t-butyl hydroperoxide (40). The absorbance at 340 nm was measured every 15 s during 5 min, and the rate of the reaction from 2 to 4 min was calculated. GPX activity was derived from a standard curve prepared by using GPX from bovine erythrocytes (Sigma). One unit of GPX activity catalyzes oxidation of 1 μmol GSH per minute at pH 7.0 and 25°C. Determinations of antioxidant enzyme concentrations are expressed per gram soluble protein assayed by the Lowry method (42).

Measurement of Plasma Aminotransferases and GGT

Blood samples were centrifuged at 4°C, and the plasma was kept cold for same-day analysis or frozen at −80°C for batch analysis. Measurement of AST, ALT, and GGT was completed by routine commercial procedures (Metpath, veterinary laboratory testing).

Data Analysis

Variables that were measured across time in the same animals, such as food intake, body weight, and concentrations of plasma AST, ALT, and GGT, were compared by means of a two-way repeated-measures ANOVA. Values on antioxidant parameters in subjects collected at discrete time points were first tested for statistically significant treatment effects by one-way ANOVA and then to a series of conditional planned comparisons and post hoc analyses. Significance was set at \( P < 0.05 \) for all comparisons by ANOVA. The familywise error for planned and post hoc comparisons was held constant at \( P = 0.016 \) for antioxidant parameters, based on three a priori comparisons (10 days of sleep deprivation vs. baseline, 5 days of sleep deprivation, and recovery after sleep deprivation), and at \( P < 0.01 \) for cell injury parameters, based on four a priori comparisons (baseline of sleep-deprived group vs. baseline of yoked group and combined baseline vs. three durations of sleep deprivation). In cases in which there was not a statistically significant difference in comparisons between like subject groups at 5 or 10 days, the two groups were combined to create a “sleep-deprived” group and a “partially sleep-deprived, yoked” group for comparison with baseline and recovery sleep groups. Nonsignificant statistical differences are designated as NS. Values are expressed as the means (SE), unless designated otherwise as the SD.

RESULTS

Physiological Variables

Food intake and body weight changes in sleep-deprived and yoked rats in noncatheterized groups in which oxidative stress parameters were determined showed a strong effect of treatment [percent change from baseline: food intake, \( F(5,38) = 5.0, P < 0.001 \); body weight, \( F(5,38) = 6.0, P < 0.001 \)] and, especially for sleep-deprived animals, a marked negative energy balance consistent with previous findings (16, 17, 22). Baseline food intake between treatment groups did not differ. Comparison of treatment conditions as a percent change from baseline values was made by calculating the percent change in daily food intake and body weight during the last 48 h of the baseline period and the last 48 h before tissue collection for each individual (Fig. 1). Sleep-deprived animals increased their food intake dramatically during the experimental period to 177% (SE 16) of basal amounts by day 10. Their food intake dropped by recovery day 2 but was still above basal amounts by 6.5% (SE 5). Sleep-deprived subjects progressively lost 16% (SE 3) of their basal body weight by recovery day 2. Yoked animals showed changes in the same direction as did sleep-deprived animals but to a lesser extent, although their food consumption was higher during the recovery period, compared with sleep-deprived animals (\( P < 0.016 \)).

Antioxidant Parameters

Glutathione content in liver showed a strong effect of experimental treatment \( F(6,37) = 2.53, P = 0.04 \). Planned comparisons of liver revealed a decrease in glutathione content of 30% at each of 5 and 10 days of sleep deprivation compared with that of baseline controls (Fig. 2A). This early and sustained decrease was markedly different from the relatively unchanged values observed in yoked animals at the same time points, which were not significantly different from basal values. Another enzymatic antioxidant in liver, catalase activity, was strongly affected by treatment \( F(6,45) = 9.97, P < 0.001 \) and was decreased in sleep-deprived rat liver from basal levels by 23% by day 5 and 36% by day 10 (Fig. 2B). Catalase activity tended to be decreased in yoked animals, but these changes were not significant. Recovery sleep was marked by return to normal concentrations of liver glutathione within 48 h...
from levels depressed by sleep deprivation. Concomitantly, catalase activity was increased to near-normal levels. The restoration of liver glutathione and catalase content was associated with marked increases in both G-6-PD, the first enzyme in the oxidative pentose phosphate pathway (Fig. 2C), and 6-PGD (Fig. 2D). The restored glutathione content in liver also was associated with a nonsignificant tendency in the direction of increased GSSG-R activity from deprivation levels and significantly increased GPX activity in the sleep-deprived group (Fig. 2, E and F). Recovery from partial sleep deprivation in the yoked animals showed evidence of high levels of activity of G-6-PD and 6-PGD that were even higher than those observed in animals recovering from total sleep deprivation. Recovery sleep in yoked animals also was associated with a significant increase in GPX activity from baseline and deprivation conditions.

In heart muscle, G-6-PD was significantly increased beyond baseline values in sleep-deprived rats by 66% at 5 days and 82% at 10 days and initially by 54% in yoked rats at 5 days of partial sleep deprivation (Fig. 3C). There were no detectable changes in total glutathione or catalase activity in heart under sleep deprivation or yoked conditions (Fig. 3, A and B). Day 10 of sleep deprivation also was associated with a 10% increase in GPX activity (Fig. 3F). Recovery sleep was marked by a strong trend (NS) toward decreased G-6-PD levels in sleep-deprived rats, which still averaged 35% above baseline, while recovering yoked animals showed evidence of a higher G-6-PD activity level of 60% above basal values. The changes in G-6-PD activity were essentially mirrored by changes in 6-PGD (Fig. 3D), although group differences from baseline control were not significant for 6-PGD under sleep-deprived or yoked conditions at 5 days. Any change in GSSG-R activity was not detectable (Fig. 3E). The sleep recovery phase in both
groups was marked by supranormal levels of GPX activity of 30 and 40% above baseline levels (Fig. 3F).

Strong treatment effects were not found for antioxidant parameters in the lung, which does not contain much glutathione compared with the liver. Changes in lung during sleep deprivation and sleep recovery were not significantly different from baseline control values for any parameter studied. Trends that did not meet statistical significance included above-basal levels of glutathione by 25% after 10 days of sleep deprivation and 30% after 2 days of recovery sleep [basal, 0.47 (SD 0.09) μmol/g wet wt] in sleep-deprived rats. The same tendencies (NS) were evident in the yoked rats, i.e., a 17% increase in glutathione after day 10 and a 40% increase after 2 days of recovery sleep. These propensities were associated with mildly enhanced lung glutathione reductase activity throughout sleep deprivation and recovery periods and enhanced G-6-PD activity on day 10 and during recovery sleep that appeared more apparent in the sleep-deprived rats, but yet they did not meet tests for statistical significance.

**Plasma Transf erase Markers of Cell Injury**

Plasma AST and ALT differed significantly between yoked and sleep-deprived animals, as shown in Fig. 4 \[ F(1,24) = 40.6, P < 0.001; F(1,24) = 9.4, P < 0.01, \] respectively. Both AST and ALT levels increased in sleep-deprived animals to 264 (SE 30) and 219% (SE 30) of baseline concentrations, respectively, without a significant change in ratio (Fig. 4, A and B). At each point across cumulative sleep deprivation, values in sleep-deprived animals were significantly different from those of yoked animals, as well as from their own baseline values. Although levels were not as high as in sleep-deprived animals, yoked animals also showed a progressive increase in plasma ALT and a late, yet significant increase in plasma AST. GGT did not differ from basal levels in either sleep-deprived or yoked animals until late, when GGT was significantly increased in sleep-deprived rats (Fig. 4C).

**DISCUSSION**

The decreases in both glutathione and catalase activity in liver by 5 days of sleep deprivation are considered to have occurred early relative to expected survival time in sleep-deprived animals and were sustained or worsened by prolongation of sleep deprivation. The 30% reductions in liver glutathione and catalase activity without increased GPX or G-6-PD activity are strong evidence that sleep deprivation produces uncompensated oxidative stress. The decreases observed in sleep-deprived rats are about as low as can be found in other experimental models of disease. For example, toxicity due to chronic cyclosporine A administration is associated with a 30–37% decrease in rat liver glutathione (33, 47). Starvation for 72 h or streptozotocin-induced diabetes in rats are each associated with 25% decreases in liver glutathione (43, 67), whereas skin scald injury-induced organ damage is associated with a 50% decrease (54). Intoxication by the herbicide paraquat is associated with a 25% decrease in liver GSH (45). Depletion of antioxidants is considered “disease-associated oxidative stress,” because decreased defenses have been associated with increased vulnerability to disease (30). Glutathione depletion of 20–30% of normal can impair cellular defense against reactive oxygen species and may lead to disrupted cell communication, aberrant protein degradation, and cell injury (41).

In the animal model of sleep deprivation, diminished antioxidant capacity occurs concomitantly with profound alterations in energy balance (Fig. 1; Refs. 2, 16, 22, and 49) and host defense impairments (21), suggesting at least two sources of oxidative burdens. Weight loss continued into the 48-h sleep recovery phase, yet antioxidant concentrations became normalized, and glutathione recycling appeared accelerated, suggesting involvement of factors beyond weight loss. Depletion of glutathione and catalase activity during sleep deprivation occurred despite a progressive rise in food consumption. The high rate of food intake during the sleep deprivation period has
been shown by other studies to be adaptive (22), and food is a major source of antioxidants. This suggests that uncompensated stress would be worse under nutritional limitations. On the other hand, oxidation of foodstuffs may produce toxic intermediaries. Even so, liver glutathione content already was diminished by the 5th day of sleep deprivation in sleep-deprived rats (Fig. 2A), at a time when food intake and body weight changes were not appreciably different from those of the partially sleep-deprived group (Fig. 1), suggesting involvement of other factors. Incomplete compensation for either a negative energy balance or immune-related activities, or yet unknown circumstances, would be expected to result in an imbalance between prooxidants and antioxidants. It is by these means that imbalances are expected to result in health impairments. Determination of the causal directions may become clearer through manipulations of diet and disease resistance.

Cell injury is expected whenever antioxidant activities are insufficient to balance oxidative stress. The present results show that sleep deprivation induced significant increases in plasma aminotransferases by day 5 that were progressive as sleep deprivation was prolonged. These results provide evidence that peripheral cell membrane damage is an early consequence of sleep deprivation, relative to advanced morbidity and lethality. Increases in both plasma AST and ALT, without a change in their ratio, suggest involvement of tissues besides the liver, such as muscle and possibly the heart. These findings echo a previous finding by Ilan et al. (32), who reported increased plasma AST and ALT concentrations in 64 human male volunteers sleep deprived for 72 h. These investigators noted being surprised and perplexed by this finding, and the causal factors were not evident. At this point, there is not an identified oxidative stress target (lipid, protein, or DNA) to directly link oxidative stress and cell injury in cause-and-effect relationships, but shared biochemical linkage between them is generally understood. Cell death by means other than oxidative stress may lead to oxidation, but decreases in major nonenzymatic antioxidants favor the view that oxidative stress is uncompensated, and this, in turn, leads to oxidant damage (further discussed in Ref. 51).

Glutathione depletion could result from inappropriately low synthesis rates or from degradation and disposal. Whereas these elements may be operating and are under study, it bears pointing out that the commonality between glutathione depletion and catalase depletion is the NADPH supply. The low-glutathione concentration in sleep-deprived animal livers was not associated with increased G-6-PD, which produces NADPH from available NADP. The lack of a G-6-PD response, together with the known metabolic and immunological burdens, suggests that maintenance of the reduced state of GSH is lost during sleep deprivation and that little of the total glutathione is available for antioxidant activities. In clinical G-6-PD deficiency, the consequential low concentrations of GSH in erythrocytes are complicated by downstream loss of normal GPX activity (reviewed in Ref. 53), which leads to increased oxidant sensitivity of the cell. It may be recalled that, in a similar manner, sleep deprivation was not associated with increased GPX activity in response to glutathione depletion. NADPH is also critical to the maintenance of catalase activity, and decreases in catalase activity can cause greater oxidant sensitivity under some circumstances than does loss of GSH (52). On the other hand, uncompensated oxidation can lead to depletion of the necessary metabolic intermediaries, such as NADH, NADPH, and ATP (51). Identification of the causal direction is under study.

Sleep recovery resulted in dramatic returns toward normal liver glutathione content and liver catalase activity in sleep-deprived animals, despite a negative energy balance and reduced food intake from deprivation levels. For both sleep-deprived and yoked groups, the increases in liver G-6-PD and 6-PGD during the recovery phase were striking. We assume that sleep restriction likely affects interruption of similar mechanisms involved in sleep processes under both partial and total sleep deprivation conditions, but to varying degrees. The differences between the recovery yoked and recovery sleep-deprived groups may be the result of the time frame of observation, and peak responses by sleep-deprived rats may have lagged or preceded those of the yoked animals. The extraordinary levels of liver G-6-PD and 6-PGD during recovery sleep suggest disinhibition of inhibitory processes during sleep deprivation and increased activities in compensation for a deficit. Unlike during sleep deprivation, the recovery sleep phase was marked by a glutathione recycling pathway that appeared to be functioning, as indicated not only by the high activity levels of G-6-PD and 6-PGD, but also by evidence of
significant increases in liver GPX and a trend toward increased GSSG-R, all associated with a return to normal liver glutathione and near-normal catalase activity.

The profile of antioxidant activity was different for the heart than for the liver. During sleep deprivation, activation of the oxidative pentose phosphate pathway was indicated by significant increases in G-6-PD and 6-PGD and maintenance of normal levels of total glutathione content and catalase activity. During 48 h of recovery sleep, the GPX activity was greatly enhanced, suggesting a response to ongoing oxidative stress and physiological flux. The heart has very little catalase and GSSG-R compared with the liver, and the heart is considered relatively devoid of antioxidant defense mechanisms (reviewed in Ref. 66). These facts accentuate the role of heart GPX during recovery sleep. The enhancement of GPX activity in heart tissue during recovery sleep suggests detoxification activities in response to sleep deprivation.

It is also possible that recovery sleep produced new oxidative burdens. Recovery sleep during the first 48 h after prolonged sleep deprivation is composed of large amounts of REM sleep (i.e., PS) (18, 23). REM sleep is a dynamic state of increased cerebral blood flow; tremendous variability of heart rate, blood pressure, and respiration; and atonia of the skeletal muscles (56a). The dynamic physiological events associated with REM are implicated in human myocardial ischemia (12, 56). This means that recovery sleep may be a state of oxidative stress even though, overall, sleep is a necessity for health and for survival. GPX activity is expected to be associated with transcription of several proteins and can be considered an adaptive response to oxidative stress.

The parameters that we measured in the lung appeared to be considerably less affected by sleep deprivation than they were in the liver or the heart. Nonsignificant tendencies were apparent for increased glutathione content in the lung, concomitant to nonsignificant tendencies for increased lung G-6-PD and 6-PGD activities and increased lung GSSG-R activity, during both sleep deprivation and recovery sleep, compared with baseline controls. In other experimental and clinical models of disease, increases rather than decreases in glutathione content in the lung are sometimes associated with pulmonary inflammatory processes, for example, during chronic lung injury in normotensive rats (36) and under some subtypes of chronic granulomatous lung infection in humans (5). Because asthma and other pulmonary diseases can be exacerbated during sleep (58) and are associated with poor sleep (24, 65), it would be premature to suggest that oxidative stress induced by sleep deprivation is not affecting protection or cellular repair in the lung. It is possible that significant oxidative stress effects are restricted to particular cell types, such as the epithelium, and selective changes may not have been detected by our whole organ procedures.

The implications of oxidative stress responses to sleep deprivation and recovery sleep are numerous. Glutathione has many roles in metabolic processes (30), including maintaining communication between cells and in intracellular transport of copper. The reduced form of glutathione is a cofactor for several enzymes in different metabolic pathways, including the leukotrienes, which are important in inflammatory reactions. Depletion of intracellular GSH is associated with immunodeficiency. Glutathione plays a role in the degradation of proteins with disulfide bonds, removal of insulin, and in protein folding.

Glutathione also is radioprotective. Vulnerability to cell injury because of decreased glutathione is amplified if antioxidant enzymes are not increased in a compensatory response (30), which occurred during sleep deprivation in our animal model.

The primary target of oxidative stress may vary, depending on the cell and on the type and severity of stress. This appears to be the reason for the diverse medical outcomes of oxidative stress-associated disease, such as atherosclerosis, rheumatoid arthritis, diabetes, neurodegeneration, cancer, and aging (30), and may provide the explanation for the many systemic conditions that are believed to be exacerbated by coexistent sleep abnormalities. Sleep after sleep deprivation is widely considered to have restorative properties, even though those properties have not been convincingly identified. The present investigation provides evidence that recovery sleep restores or accentuates antioxidants and antioxidant activities in the heart and the liver.

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