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Antioxidant responses to chronic hypoxia in the rat cerebellum and pons

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Abstract

Obstructive sleep apnea (OSA) is characterized by chronic intermittent hypoxia (CIH) and sleep fragmentation and deprivation. Exposure to CIH results in oxidative stress in the cortex, hippocampus and basal forebrain of rats and mice. We show that sustained and intermittent hypoxia induces antioxidant responses, an indicator of oxidative stress, in the rat cerebellum and pons. Increased glutathione reductase (GR) activity and thiobarbituric acid reactive substance (TBARS) levels were observed in the pons and cerebellum of rats exposed to CIH or chronic sustained hypoxia (CSH) compared with room air (RA) controls. Exposure to CIH or CSH increased GR activity in the pons, while exposure to CSH increased the level of TBARS in the cerebellum. The level of

TBARS was increased to a greater extent after exposure to CSH than to CIH in the cerebellum and pons. Increased superoxide dismutase activity (SOD) and decreased total glutathione (GSht) levels were observed after exposure to CIH compared with CSH only in the pons. We have previously shown that prolonged sleep deprivation decreased SOD activity in the rat hippocampus and brainstem, without affecting the cerebellum, cortex or hypothalamus. We therefore conclude that sleep deprivation and hypoxia differentially affect antioxidant responses in different brain regions.

Keywords: antioxidant responses, cerebellum, chronic intermittent hypoxia, chronic sustained hypoxia, pons, sleep deprivation.

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Obstructive sleep apnea (OSA) is a condition characterized by repeated upper airway obstruction during sleep. It affects ~4% of adult men and 2% of women (Young *et al.* 1993; Loredó *et al.* 2001). OSA causes neurocognitive and behavioral deficits including excessive daytime sleepiness, impaired short-term memory, and problems with language comprehension and expression, all of which are compatible with global executive dysfunction (Bedard *et al.* 1993; Naegele *et al.* 1998; Gozal *et al.* 2001; Beebe and Gozal 2002). OSA also leads to increased cardiovascular morbidity, including hypertension, cardiovascular and cerebrovascular disease and increased risk for sudden death (Zaninelli *et al.* 1991; Seppala *et al.* 1991; Fletcher *et al.* 1992; Lavie *et al.* 1993; Fletcher 1995; Silverberg and Oksenberg 1997; Greenberg *et al.* 1999). Furthermore, magnetic resonance imaging studies in patients with OSA revealed significant gray matter loss in several brain regions including the cortex, hippocampus and cerebellum, suggesting that sleep apnea might result in neuronal

damage, impairing cognitive function (Macey *et al.* 2002). Of note, localized gray matter loss was also seen unilaterally in normally well-perfused regions involved in motor regulation of the upper airway, suggesting that underlying brain damage may also precipitate the occurrence of OSA in some of these patients.

OSA produces both chronic intermittent hypoxia (CIH) and sleep fragmentation and deprivation. Rats subjected to

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Abbreviations used: CIH, chronic intermittent hypoxia; CSH, chronic sustained hypoxia; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GR, glutathione reductase; GSht, total glutathione; GSSG, oxidized glutathione; GSH, reduced glutathione; OSA, obstructive sleep apnea; RA, room air; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

CIH, of similar magnitude to that experienced by sleep apnea patients, showed time-dependent increases in neuronal apoptosis within the CA1 hippocampal region and neocortex (Gozal *et al.* 2001). Based on these findings, it was proposed that hypoxia-induced damage might result from oxidative stress. Oxidative stress occurs when free radicals are produced at a greater rate than they are removed, resulting not only in changes in the activities of antioxidative enzymes and the levels of endogenous antioxidants, but ultimately in oxidative damage to lipids, proteins and/or nucleic acids. As further evidence of the viability of the oxidative stress hypothesis, Row *et al.* (2003) reported that the antioxidant PNU-101033E significantly attenuated the behavioral and oxidative changes associated with CIH (Row *et al.* 2003). Transgenic mice over-expressing Cu/Zn-superoxide dismutase (SOD1; EC 1.15.1.1) showed lower cortical free radical production and reduced apoptosis compared with normal mice exposed to CIH (Xu *et al.* 2004). Veasey *et al.* (2004) recently showed that 8 weeks of CIH increased lipid oxidation, protein oxidation and mRNA levels of several antioxidant enzymes in the rostral basal forebrain. CIH also increased iNOS mRNA and protein expression, as well as NOS activity, nitrotyrosine residue formation and nitrate/nitrite production (Li *et al.* 2004). We previously reported that prolonged sleep deprivation (5–11 days) decreased Cu/Zn-SOD activity in the rat hippocampus and brainstem (Ramanathan *et al.* 2002). This finding is consistent with the hypothesis that enzymatic damage results from oxidative stress, whereby the overproduction of free radicals decreases SOD activity (Davies *et al.* 1987; Yunoki *et al.* 1998).

In this study, we investigated the effects of hypoxia on antioxidant responses, as an indicator of oxidative stress, in the rat cerebellum and pons. The cerebellum is of particular interest, because of its role in respiratory and autonomic control (Xu and Frazier 2002), while the pons is involved in sleep–wake regulation and also in hypoxia-induced hypothermia and hyperventilation (Fabris *et al.* 1999).

Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which are always present in cells due to normal metabolism, are removed by endogenous antioxidative enzymes and antioxidants. There are many enzymes that contribute significantly to the antioxidant responses. In this study we measured the activities of Cu/Zn-SOD, glutathione reductase (GR; EC 1.8.1.7) and total glutathione (GSht) levels. We also analyzed the levels of the lipid oxidation product, thiobarbituric acid reactive substances (TBARS). Changes in these markers reflect changes in the levels of free radicals, and are therefore indicative of oxidative stress.

Materials and methods

Young adult male Sprague–Dawley rats (45–50 days of age) were used for all experiments. The experimental protocols were approved by our Institutional Animal Use and Care Committee and conform

to the National Institutes of Health guide for the care and use of laboratory animals.

Hypoxic exposures

Rats were randomly assigned to three experimental groups consisting of: (i) CIH ($n = 6$ /time point); (ii) CSH ($n = 6$ /time point) for 6 h, 1, 3, 7, 14 or 30 days; or (iii) room air (RA, $n = 6$). Animals were placed in four identical commercially designed chambers ($30 \times 20 \times 20$ in.; Oxycycler model A44XO; Biospheryx, Redfield, NY, USA). The chambers were operated under a 12:12 h light/dark cycle (light 06.00 to 18.00). 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 12 h of light time by a computerized system controlling the gas valve outlets. Deviation from the desired concentration was corrected by addition of N_2 or O_2 through solenoid valves. For the remaining 12 h of night, oxygen concentrations were kept at the RA concentration of 21%. Ambient CO_2 in the chamber was periodically monitored and maintained at $< 0.01\%$ by adjusting overall chamber basal ventilation. Hypocapnic hypoxia models most aspects of the OSA syndrome (Gozal *et al.* 2001). The gas was circulated through a molecular sieve (type 3A; Scientific Instrument Services, Ringoes, NJ, USA) to remove ammonia. Humidity was measured and maintained at 40–50% by circulating the gas through a freezer and silica gel. Ambient temperature was kept at 22–24°C. The CIH profile consisted of exposing rats to alternating RA and 10% O_2 every 90 s during the light phase (06.00 to 18.00). The CSH profile consisted of exposing rats to 10% oxygen throughout the light phase. The rats were exposed to RA for the remaining 12 h (18.00 to 06.00). Hence, the CIH group was re-oxygenated every 90 s during the light phase, while the CSH group was re-oxygenated at the onset of the light phase and then again at the onset of the dark phase. Rats exposed to RA throughout the experimental time period served as controls.

Biochemical analysis

Animals were killed at the end of the designated duration of hypoxia exposure and the cerebellum and pons were dissected on ice and stored at $-80^\circ C$ until analyzed. The cerebellum and pons were divided into two portions, one portion was homogenized in a hand-held homogenizer with 12 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 (5415C) at 320 g for 10 min at 4°C. The pellet was discarded and the supernatant re-centrifuged at 14 000 g for 30 min at 4°C. This supernatant was used for determining the activities of Cu/Zn-SOD, GR and the levels of GSht. The remaining portion of the cerebellum and pons was homogenized with 12 strokes in cold homogenizing buffer containing 20 mM phosphate buffer (pH 7.4) to which 1% of 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 2900 g for 10 min at 4°C. The pellet was discarded and the supernatant was used for determining the level of the lipid oxidation product, TBARS.

Prior to determining the enzyme activities and the level of GSht and TBARS, the protein content of the samples was determined with

a protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) using bovine plasma gamma globulin 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 activity

SOD activity was measured according to the method of Misra and Fridovich (1972). 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 auto-oxidation of epinephrine was measured at 480 nm for 180 s on a Hitachi U2000 spectrophotometer. SOD activity was expressed as units (U) of SOD/mg of protein, where 1 U of SOD is defined as the amount of enzyme present that inhibits the auto-oxidation of epinephrine by 50%.

Glutathione reductase activity

GR activity was determined according to the method of Somani and Husain (1997). Tissue extract was added to the reaction mixture containing phosphate buffer (50 mM), 50 μ L of GSSG (20 mM) and 100 μ L of NADPH (2 mM in 10 mM Tris-HCl pH 7.0). The rate of NADPH oxidation was measured at 340 nm for 180 s. The molar extinction coefficient of 6.22×10^3 (Mcm)⁻¹ was used to determine GR activity. GR activity was expressed as mM NADPH oxidized/min/mg protein.

Total glutathione levels

GSht was measured by the enzymatic recycling procedure in which GSH is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to GSSG, which is then reduced by NADPH in the presence of GR back to GSH (Griffith 1980). To 800 μ L of NADPH (0.3 mM) and 100 μ L of DTNB (6 mM), 100 μ L of either tissue extract or known amounts of GSH standard were added. The reaction was initiated with 10 μ L of GR (50 units/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 until it exceeds a value of 2.0 (~ 120 s). Total glutathione levels were expressed as nmoles GSH/g tissue.

Lipid oxidation assay

Lipid oxidation was determined as TBARS according to the method of Ohkawa *et al.* (1979). Tissue extract (100 μ L) was added to a mixture containing 50 μ L of sodium dodecyl sulfate (8.1%), 375 μ L of acetic acid (20%, pH 3.5) and 375 μ L 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 1300 g for 10 min. The colored upper organic layer was removed and read at 532 nm. Tetraethoxypropane was used as an external standard, and the level of lipid peroxides was expressed as nmoles malondialdehyde/mg protein.

Statistical analysis

Each type of assay was carried out on a different day, and so in order to minimize the differences due to sample and/or reagent preparation, we normalized the data to the mean of each set. For each assay

(SOD, GR, GSht and TBARS), values from duplicate samples were averaged to obtain 1-value point. The specific SOD activity for each sample was first calculated as USOD/mg protein. Each assay set was run at one time and included 13 samples: RA (1 sample), CIH (6 samples) and CSH (6 samples). Each sample from a particular assay set was normalized by dividing the specific SOD activity of that sample by the mean specific SOD activity of the assay set. The average of six assay sets was then used to determine the final normalized specific SOD activity for each sample. The results are expressed as fold increase in SOD activity \pm SEM. The activity of GR as well as the levels of GSht and TBARS, were analyzed and expressed in the same way. Two-way analyses of variance (treatment and time) with post-hoc Bonferroni analysis were used to determine statistical significance, which was set at a level of $p < 0.05$.

Results

There was a significant effect of time ($F_{6,77} = 6.17$, $p < 0.001$) and treatment ($F_{1,77} = 8.13$, $p < 0.05$) on the level of TBARS in the cerebellum of rats exposed to hypoxia (Fig. 1a). TBARS was significantly higher after 1 day (42%, $t = 2.90$, d.f. = 5, $p < 0.05$), 7 days (39%, $t = 2.72$, d.f. = 5, $p < 0.05$), 14 days (65%, $t = 4.51$, d.f. = 5, $p < 0.001$) and 30 days (69%, $t = 4.78$, d.f. = 5, $p < 0.001$) of exposure to CSH compared with RA controls. There was no significant time difference in the amount of TBARS in rats exposed to CIH compared with RA controls. Also a greater amount of TBARS was produced in the cerebellum of rats exposed to 1 day (26%, $t = 2.03$, d.f. = 5, $p < 0.05$), 14 days (26%, $t = 2.37$, d.f. = 5, $p < 0.05$) and 30 days (30%, $t = 2.72$, d.f. = 5, $p < 0.01$) of CSH compared with CIH (Fig. 1a). There was a significant interaction between time and treatment on the level of TBARS in the pons ($F_{6,77} = 2.35$, $p < 0.05$). In other words, the effect of different levels of time depended on what level of treatment was present. TBARS levels were significantly higher in CSH animals than CIH animals after 30 days of exposure (24%, $t = 3.15$, d.f. = 5, $p < 0.01$, Fig. 1b).

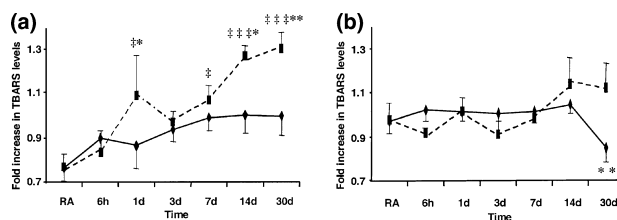


Fig. 1 Thiobarbituric acid reactive substance (TBARS) levels in the (a) cerebellum and (b) pons of rats exposed to room air (RA, $n = 6$), chronic intermittent hypoxia (CIH, diamonds, $n = 6$ /time point) or chronic sustained hypoxia (CSH, squares, $n = 6$ /time point). Bars represent standard error. (\ddagger ; $p < 0.05$, $\ddagger\ddagger\ddagger$; $p < 0.001$, CSH vs. RA; \ast ; $p < 0.05$, $\ast\ast$; $p < 0.01$ CSH vs. CIH, post-hoc Bonferroni.) Normalized RA values for cerebellum = 0.77 ± 0.06 and pons = 0.97 ± 0.06 .

There was a significant effect of time ($F_{6,77} = 3.39$, $p < 0.005$) but not of treatment ($F_{1,77} = 0.8$, $p > 0.05$) on GR activity in the pons (Fig. 2a). GR activity was significantly higher after 14 days (37%, $t = 3.09$, d.f. = 5, $p < 0.05$) of exposure to CIH, and after 6 h (34%, $t = 2.85$, d.f. = 5, $p < 0.05$) and 1 day (35%, $t = 2.85$, d.f. = 5, $p < 0.05$) of exposure to CSH compared with RA controls (Fig. 2a). There was no significant effect of either time (compared with RA) or treatment (CIH vs. CSH) on GR activity in the cerebellum.

There was a significant effect of treatment ($F_{1,77} = 5.81$, $p < 0.05$) but not time ($F_{6,77} = 1.36$, $p > 0.05$) on SOD activity in the pons (Fig. 2b). SOD activity was significantly higher after 6 h of exposure to CIH compared with CSH (21%, $t = 2.60$, d.f. = 5, $p < 0.01$). There was no significant effect of either time or treatment on SOD activity in the cerebellum.

Similarly, there was a significant effect of treatment ($F_{1,77} = 20.29$, $p < 0.001$) but not time ($F_{6,77} = 2.13$, $p > 0.05$) on GSht levels in the pons (Fig. 2c). GSht levels were significantly lower after 6 h (15%, $t = 2.29$, d.f. = 5, $p < 0.05$), 1 day (15%, $t = 2.12$, d.f. = 5, $p < 0.05$), 3 days (15%, $t = 2.22$, d.f. = 5, $p < 0.05$) and 14 days (20%, $t = 3.42$, d.f. = 5, $p < 0.001$) of exposure to CIH compared with CSH (Fig. 2c). There was no significant effect of either time or treatment on GSht levels in the cerebellum.

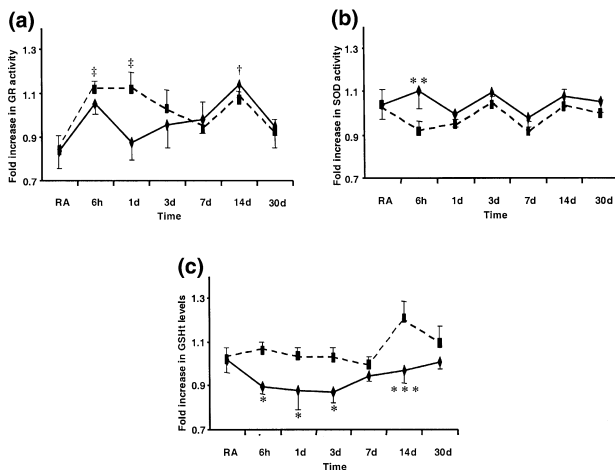


Fig. 2 (a) Glutathione reductase (GR) activity (b) superoxide dismutase (SOD) activity and (c) total glutathione (GSht) levels in the pons of rats exposed to room air (RA, $n = 6$), chronic intermittent hypoxia (CIH, diamonds, $n = 6$ /time point) or chronic sustained hypoxia (CSH, squares, $n = 6$ /time point). Bars represent standard error. ($‡p < 0.05$, CSH vs. RA; $†p < 0.05$, CIH vs. RA, post-hoc Bonferroni.) ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, CSH vs. CIH, post-hoc Bonferroni). Normalized RA values for GR activity = 0.83 ± 0.07 , SOD activity = 1.04 ± 0.07 and GSht levels = 1.02 ± 0.06 .

Discussion

In this study we show that hypoxia, at levels commonly occurring in human patients with sleep apnea, induces antioxidant responses in the rat cerebellum and pons. This is indicated by increased activities of the antioxidant enzymes, SOD and GR, decreased levels of the endogenous antioxidant, GSht, and elevated levels of the lipid oxidation product, TBARS.

Hypoxia significantly increased GR activity in the pons and TBARS levels in the cerebellum compared with RA controls. The increased GR activity indicates an increase in the levels of hydrogen peroxide (H_2O_2), which may arise from: (i) the reaction of superoxide ($O_2^{\bullet-}$) with superoxide dismutase; (ii) as a normal byproduct of the activity of several enzymes, including monoamine oxidase, tyrosine hydroxylase and L-amino oxidase; or (iii) via the autoxidation of endogenous substances, such as ascorbic acid and catecholamines. The increased levels of H_2O_2 , if not removed by GR, will ultimately lead to increased levels of the lipid oxidation product, TBARS, which is observed in the cerebellum. Increased H_2O_2 levels could also elevate levels of oxidized proteins and/or nucleic acids, which may have been altered in the pons, but were not investigated in this study.

Cerebellar structures play an essential role in the onset of inspiration after apnea (Lutherer and Williams 1986; Gozal *et al.* 1995), in recovery from extremes of blood pressure changes (Lutherer *et al.* 1983), in carbon dioxide regulation (Xu and Frazier 1997) and in gasping (Smejkal *et al.* 1999; Peiffer *et al.* 2001). Macey *et al.* (2002) speculated that the cerebellar damage, linked to disordered breathing during sleep, results from the inappropriate coordination of upper airway muscles. Bennett *et al.* (2003) showed that perinatal asphyxia, resembling the clinical situation of intrauterine hypoxia-ischemia, caused neurodegeneration, neuronal loss and gliosis in the cerebellum of the adult guinea pig. Hypoxia also caused a significant increase in Fos-immunoreactivity in several pontine nuclei of fetal sheep (Breen *et al.* 1997). In addition, Fabris *et al.* (1999) showed that nitric oxide in the locus coeruleus was responsible for the inhibitory modulation of the ventilatory response to hypoxia in adult rats.

Hypoxia differentially affects antioxidant responses in the cerebellum and pons across the period observed. Regional differences in antioxidant responses have similarly been observed after sleep deprivation (Ramanathan *et al.* 2002). OSA produces both CIH as well as sleep fragmentation and deprivation. We compared antioxidant responses due to sleep deprivation with that produced by CIH and CSH (Table 1). We show that hypoxia and sleep deprivation differentially affect antioxidant responses in different brain regions and may interact in the progression of OSA.

Furthermore, CSH significantly increased the levels of TBARS compared with CIH in the cerebellum. However,

Table 1 Antioxidant responses in different brain regions as a result of prolonged (5–11 days) sleep deprivation (SD), chronic intermittent hypoxia (CIH) or chronic sustained hypoxia (CSH)

	SD	CIH	CSH
Brainstem	decreased SOD activity	increased GR activity	increased GR activity
Hippocampus	decreased SOD activity	increased RNA damage	not done
Cerebellum	no change	no change	increased TBARS
Cortex	no change	increased MDA and isoprostane	not done
Hypothalamus	no change	not done	not done

CIH significantly decreased GSht levels compared with CSH in the pons. Several studies have shown decreased GSht content under various oxidative stress conditions, such as after repeated inhalation exposure to manganese sulfate (Dobson *et al.* 2003), hypoxia–reoxygenation induced damage to astroglia-rich cells (Makarov *et al.* 2002) and after 96 h of REM sleep deprivation (D’Almeida *et al.* 1998).

The differences in antioxidant responses as a function of region, time and treatment may be due to: (i) how well the brain areas are perfused, (ii) different brain areas being differentially resistant to hypoxia, (iii) different brain areas being more resistant to CIH than CSH, and/or (iv) different antioxidant capacity of different brain areas. These antioxidant responses could initiate a positive feedback loop whereby hypoxia causes cellular damage which then produces further respiratory and sleep/wake impairment. In particular, the CIH profile producing blood oxygenation changes comparable with those seen in sleep apnea, may explain the brain damage and cognitive effects seen in the sleep apnea syndrome.

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