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Manganese superoxide dismutase protects mouse cortical neurons from chronic intermittent hypoxia-mediated oxidative damage

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Obstructive sleep apnea (OSA) syndrome has been recognized as a highly prevalent public health problem and is associated with major neurobehavioral morbidity. Chronic intermittent hypoxia (CIH), a major pathological component of OSA, increases oxidative damage to the brain cortex and decreases neurocognitive function in rodent models resembling human OSA. We employed *in vitro* and *in vivo* approaches to identify the specific phases and subcellular compartments in which enhanced reactive oxygen species (ROS) are generated during CIH. In addition, we utilized the cell culture and animal models to analyze the consequences of enhanced production of ROS on cortical neuronal cell damage and neurocognitive dysfunction. In a primary cortical neuron culture system, we demonstrated that the transition phase from hypoxia to normoxia (NOX) during CIH generates more ROS than the transition phase from NOX to hypoxia or hypoxia alone, all of which generate more ROS than NOX. Using selective inhibitors of the major pathways underlying ROS generation in the cell membrane, cytosol, and mitochondria, we showed that the mitochondria are the predominant source of enhanced ROS generation during CIH in mouse cortical neuronal cells. Furthermore, in both cell culture and transgenic mice, we demonstrated that overexpression of MnSOD-decreased CIH-mediated cortical neuronal apoptosis, and reduced spatial learning deficits measured with the Morris water maze assay. Together, the data from the *in vitro* and *in vivo* experiments indicate that CIH-mediated mitochondrial oxidative stress may play a major role in the neuronal cell loss and neurocognitive dysfunction in OSA. Thus, therapeutic strategies aiming at reducing ROS generation from mitochondria may improve the neurobehavioral morbidity in OSA.

Keywords: Sleep apnea; Chronic intermittent hypoxia; Oxidative stress; Reactive oxygen species; MnSOD; Neurocognitive dysfunction

Introduction

Obstructive sleep apnea (OSA) syndrome, characterized by episodic cessation of airflow during sleep, has been recognized as a highly prevalent public health problem affecting 2% of women and 4% of men in the general US population. Chronic intermittent hypoxia (CIH) is a major pathological element of OSA that Consequently decreases neurocognitive function and increases cardiovascular morbidity in patients with OSA (Young et al., 2002a,b; Naegele et al., 1995). More recently, brain structural abnormalities have been detected in OSA patients using functional MRI approaches (Macey et al., 2002, 2006). The significant brain gray matter loss found in OSA patients is apparently associated with alterations in neural circuitry and neurocognitive function (Macey and Harper, 2005; Woo et al., 2005). Similarly, animal models mimicking the intermittent hypoxic episodes of OSA elicit hippocampal and cortical neuronal cell damage, and consequently result in deficits in spatial task learning and working memory (Xu et al., 2004; Gozal et al., 2001, 2003). However, the cellular and molecular processes underlying the neuronal cell loss and consequent neurocognitive dysfunction remain incompletely delineated.

Reactive oxygen species (ROS) including superoxide, hydroxyl radicals, hydrogen peroxide, and peroxynitrite are the oxygen-containing molecules that are highly reactive with proteins, lipids and nucleic acids. They have been identified as perpetrators of cell death, tissue damage and functional alterations in human neurodegenerative diseases. Evidence has accumulated in recent years to suggest that increased oxidative stress is associated with neurocognitive and cardiovascular dysfunction in human OSA (Lavie et al., 2004; El Solh et al., 2006; Grebe et al., 2006; Carpagnano et al., 2003). The oscillations in oxygen concentration during CIH are reminiscent of the process of ischemia (hypoxia)/re-oxygenation, and may contribute to the increased production of ROS (Prabhakar et al., 2006; Prabhakar and Kumar, 2004; Lavie, 2003, 2005). In support of this assumption, we have previously shown in rats that systemic administration of an electron spin

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trapper abrogated CIH-induced deficits in the acquisition of a spatial task in the water maze (Row et al., 2003). Furthermore, the excessive somnolence that develops following CIH exposures in mice appears to be mediated at least in part by oxidative stress-induced cellular losses within the locus coeruleus, and is markedly reduced in NADPH oxidase deficient mice (Zhan et al., 2005; Veasey et al., 2004). Although increased oxidative stress has been causally linked to neuronal cell losses in a variety of neurodegenerative diseases, the cellular and molecular mechanisms of CIH-induced brain structural changes and consequent neurophysiological alterations remain largely undefined. In addition, the specific phase(s) and sub-cellular compartment(s) where increased ROS production occurs remain to be determined. To this end, we utilized cell culture and transgenic mouse models to analyze CIH-mediated oxidant generation, CIH-induced neuronal apoptosis, and CIH-mediated neurocognitive dysfunction.

Methods

Chemicals and reagents

Rotenone, allopurinol, apocynin, and 5,5-dimethyl pyrroline-*N*-oxide (DMPO) were purchased from Sigma Chemicals (St. Louis, MO). 2′,7′-Dicholorodihydrofluorescein diacetate (DCF), and di-hydroethidine (DHE) were purchased from Molecular Probes (Eugene, OR).

Neurobasal medium, fetal bovine serum (FBS), B27 and glutamine were purchased from Invitrogen Inc. (Carlsbad, CA).

Animals

C57BL/6J mice (Stock # 000664) (male, 12 weeks of age, bodyweight 22–25 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Transgenic mice overexpressing MnSOD were provided by Dr. Daret St. Clair (University of Kentucky) (Yen et al., 1996, 1999). The experimental protocols used for breeding and CIH experiments were approved by the Institutional Animal Use and Care Committee and are in close agreement with the National Institutes of Health guidelines for the care and use of laboratory animals. All efforts were made to minimize animal stress and to reduce the number of animals used.

Primary cortical neuronal cell culture

Primary cortical neuronal cultures were prepared and maintained according to a previously described method with minor modifications (Brewer, 1995). Briefly, the cerebral cortical regions from embryonic day 15.5 (E15.5) mouse fetuses were dissected out and rinsed twice with ice-cold Ca^{2+}/Mg^{2+}-free Hanks' salt solution (HSS), pH 7.5. After removal of meninges, cells were dissociated by trituration through a 5-ml pipette first and then a flame-polished Pasteur pipette. Cells were spun down by centrifugation and resuspended in Neurobasal medium supplemented with 0.2% heat-inactivated fetal bovine serum (FBS), 2% B27, 1.2 mM glutamine and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Dissociated cells were then either plated in 35-mm dishes or in chamber slides (Corning Life Sciences, NY) that were coated with poly-L-lysine (0.1 mg/ml). The neurons were grown in a humidified atmosphere of 5% CO_2–95% air at 37 °C for 3–4 days and were used for experiments in Neurobasal medium containing 2% B27 and 0.2% FBS.

Chronic intermittent hypoxic conditions for primary cortical neurons

Cultured cortical neurons were either exposed to normoxia (NOX: 21% O_2, 5% CO_2, and balance N_2), or to CIH (Episodic cycles of NOX: 21% O_2, 5% CO_2, and balance N_2 for 25 min; and hypoxia: 0.1% O_2, 5% CO_2, and balance N_2 for 35 min), using a custom-designed, computer-controlled incubation chamber attached to an external O_2–CO_2 computer-driven controller (Biospherix, Redfield, NY). Chamber O_2, N_2, and CO_2 levels were continuously monitored and adjusted according to the desired programmed profile. In addition, O_2 content in the medium was monitored with a fiber optic O_2 sensor (Ocean Optics, Dunedin, FL) placed 1 mm above the cell layer to ensure the specific experimental profile implementation. O_2 levels in the air phase as well as in the medium were continuously monitored O_2 sensor during CIH (Ocean Optics, Dunedin, FL).

Chronic intermittent hypoxic conditions for experimental mice

Exposures of mice to CIH were carried out as previously described (Xu et al., 2004). Briefly, animals were housed in a chamber (30 in.×20 in.×20 in.) and were operated under 12-h light–dark cycle (Oxycycler model A44XO, Biospherix, Redfield, NY). Gas was circulated around the chamber at 60 L min^{-1} (i.e., one complete change per 10 s). The O_2 concentration was continuously measured by an O_2 analyzer, and was changed throughout the 12 h of light time (06:00 a.m. to 6:00 p.m.) by a computerized system controlling the gas valve outlets, such that the moment to moment desired oxygen concentration of the chamber was adjusted automatically. This system permitted delineation of oxygenation profiles in the mouse model that mimic those of patients with OSA. For all of the experiments, the CIH profiles consisted of alternating NOX (21% O_2) and hypoxia (5.7% O_2) every 90 s. Deviations from the desired O_2 concentrations were supplied by addition of N_2 or room air through solenoid valves. Ambient CO_2 in the chamber was periodically monitored and maintained at 0.03% by modifying the ventilation of the chamber. Humidity was measured and maintained at 40–50% and temperature was kept at 22–24 °C. The selection of this CIH profile emanated from a series of preliminary experiments in which the magnitude of hypoxia, and length of hypoxia and NOX were tested and confirmed to reproduce the respiratory patterns and neurocognitive deficits in the mouse model (Tagaito et al., 2001; Polotsky et al., 2006). The durations of CIH exposures were from 1 day to 30 days. NOX control mice were placed in neighboring chambers but were exposed to normoxic gas. At least three mice per group were used for the specific studies.

At the end of the designated duration of CIH exposures, mice were anesthetized with pentobarbital (50 mg/kg) and perfused with 0.9% saline buffer. Mouse brain cortical regions were dissected out, frozen in liquid nitrogen immediately and stored in a −80 °C freezer until analysis.

Dicholorodihydrofluorescein (DCF) and di-hydroethidine (DHE) oxidation assays of CIH-mediated ROS production in primary cortical neurons

The production of ROS was measured spectrofluorometrically by using the probe of 2′,7′-dicholorodihydrofluorescein diacetate (DCF), or di-hydroethidine (DHE). Briefly, 6×10^4 cortical neurons (final number of neuronal cells in each well) were cultured in