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ScienceDirect
Life Sciences 79 (2006) 1638–1644
Life Sciences
www.elsevier.com/locate/lifescie

Evidence for an age-related attenuation of cerebral microvascular antioxidant response to oxidative stress

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Received 31 December 2005; accepted 23 May 2006

Abstract

Effects of aging and oxidative stress were studied in cerebral microvessels and microvessel-depleted brain from 6-, 18-, and 24-month-old C57Bl/6J mice exposed to normoxia, 24 or 48 h hyperoxia, or 24 h hyperoxia followed by 24 h normoxia. Microvessels lacked smooth muscle and consisted predominantly of endothelium. Following exposure and isolation of microvessel and parenchymal proteins, Western blot analysis was performed for detection of cytosolic thioredoxin 1 (TRx 1) and mitochondrial thioredoxin 2 (TRx 2), protein carbonyl, and mitochondrial superoxide dismutase (MnSOD). Both microvessel and parenchymal TRx 1 levels were increased by hyperoxia; however, the microvascular response was limited and delayed in comparison to that of the parenchymal fraction. Whereas TRx 2 levels in microvessels were increased in older mice, irrespective of exposure condition, hyperoxia per se had little or no apparent effect. Parenchymal cells showed no age-related increase in TRx 2 level under normoxic conditions, but showed increased levels following hyperoxia. Microvessel MnSOD was lower than that in parenchymal cells, but increased with age under normoxia, and also was correlated with the duration of hyperoxia. Although hyperoxia augmented MnSOD levels in young (6 months) and middle-aged (18 months) animals, the response was less pronounced in microvessels from senescent, 24-month-old mice. Unlike microvessels, which showed a sustained age-related increase in MnSOD level under each exposure condition, parenchymal cells from normoxic mice showed no increase, and hyperoxia-induced elevations declined with prolonged 48 h exposure. These results indicate that the microvessel endothelium is (1) subjected to a more intense oxidative environment than neurons and glia and (2) is limited by aging in its ability to respond to oxidative insult.

Keywords: Hyperoxia; Oxidative stress; Microvessel; Mouse; Aging; Brain

Introduction

Reactive oxygen (ROS) molecular species normally are produced by cellular metabolism and contribute importantly to regulation of intracellular oxidation–reduction (redox) homeostasis (Hensley and Floyd, 2002). Cellular dysfunction associated with aging in mammalian species is attributable, in part, to an increased production of ROS and alterations in antioxidant capacity (Sohal and Orr, 1992; Mo et al., 1995; Kim et al., 2003; Kasapoglu and Özben, 2001). A result of this oxidative stress is an accelerated rate of oxidatively modified proteins and lipids (Çakatay et al., 2001; Onorato et al., 1998). Although generation of ROS occurs at multiple sites within the cell, mitochondrial electron transport is considered to be the primary generator of ROS, and therefore the principal contributor to oxidative stress within the cell (Floyd et al., 2001). Both cytosolic and mitochondrial compartments possess antioxidant repair systems that reduce oxidized amino acid residues in proteins and reduce both protein and lipid carbonyls. The thioredoxin/thioredoxin reductase system that reduces oxidatively modified protein cysteine residues is critical to the maintenance of redox balance (Takagi et al., 1999; Lovell et al., 2000). Thioredoxins are upregulated as an antioxidant response to oxidative stress within mammalian cells, and thus can modulate cellular redox status directly by thiol reduction (Deneke, 2000). Thioredoxin may also affect expression of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) in response to oxidative stress, including that induced by hyperoxia [Das et al., 1997].

The cerebral microvessel endothelium is continuously exposed to a high oxygen partial pressure (PaO_2), which may predispose these cells to an injurious level of ROS. In the present study, we investigate the effect of normobaric hyperoxia-

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0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.05.018

induced oxidative stress and aging on the antioxidant response of cerebral microvessels and parenchymal cells isolated from young adult (6 months), middle-aged (18 months) and senescent (24 months) C57Bl/6J mice. The results of this study show activation of an acute microvascular response to oxidative stress that is attenuated with senescence, whereas brain parenchymal cells (neurons and glial cells) are less affected by aging and hyperoxia with respect to induction of antioxidant response.

Materials and methods

Chemicals

MES buffer [2-(*N*-morpholino)ethanesulfonic acid, monohydrate, pK_a 6.15] was purchased from Calbiochem (La Jolla, CA). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). The following primary antibodies were used: polyclonal antibodies to Trx 1 and Trx 2 (gift from Dr. Sue Goo Rhee, NHLBI, NIH), anti-MnSOD rabbit polyclonal (cat. RDI-RTSODMAbR) from RDI (Flanders, NJ); and anti-β-actin (AC-74) mouse monoclonal (cat. A-5316) from Sigma (Flanders, NJ). Protein carbonyl detection was performed according to the manufacturer's directions using an Oxyblot kit from Intergen (Purchase, NY). Secondary antibodies (HRP-conjugated), included anti-mouse IgG (cat. NA 931) and anti-rabbit IgG (cat. NA 9340) from Amersham Life Science (Little Chalfont, UK). Antibody detection was performed using enhanced chemiluminescence detection reagents (ECL) purchased either from Amersham Life Science, or BioRad Laboratories (Hercules, CA). All reagents for Western blot analysis were purchased from Invitrogen (Carlsbad, CA).

Experimental animals

The C57Bl/6J mice used for this study were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, Indiana), through the National Institutes of Health, NIA, Aging colony. Throughout the study all animals were given access to food and water ad libitum, and housed in a room with a 12 h on/off lighting cycle. Maintenance and experimental procedures to which mice were subjected adhered to *ALAC*-approved guidelines and guidelines described in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23).

Exposure conditions and microvessel isolation

Mice 6, 18, and 24 months of age were maintained under ambient air (normoxia) or were exposed to 100% oxygen (normobaric hyperoxia) for 24 or 48 h within a chamber equipped with a 12 h on–off lighting cycle, and where food and water were supplied ad libitum. An additional group of mice was exposed to hyperoxia for 24 h and then allowed to survive for 24 h before sacrifice (normoxic recovery). Animals were subsequently euthanized by cervical dislocation, and the brains removed for homogenization and isolation of cerebral microvessels, as described previously (Williams et al., 1988). For each age and exposure condition, the cerebral cortex and midbrain from 10 to 20 animals were pooled and homogenized in 0.4 M sucrose solution containing 0.1 mM $CaCl_2$, 0.4 mM MES buffer, 0.1 mM ATP, 1% BSA, and 20,000 IU sodium heparin at 4 °C. The protease inhibitors aprotinin (0.5 μg/ml), leupeptin (0.5 μg/ml), and pepstatin (0.7 μg/ml) were added to the above isolation solution to inhibit protease activity (Levine et al., 1990) and 0.1 mM EDTA added as metal chelator. Contamination by myelin was eliminated by centrifuging the homogenate in sucrose containing 15% dextran. The homogenate was subsequently passed through nylon mesh filters attached to glass bead columns. The resulting microvessel and microvessel-depleted brain parenchymal fractions were stored at −70 °C in 0.4 M sucrose solution without BSA until prepared for gel electrophoresis and Western blotting. The microvessel fraction consisted predominantly of capillaries and venules (vessel dia. ~10 μm) with infrequent contamination from small arterioles (vessel dia. ~30 μm), and no observable contamination by intact parenchymal cells, as determined by light microscopic examination of each isolate. The isolation procedure used for separating microvessels from brain parenchymal cells resulted in the elimination of intravascular blood cells and an isolate of consistently high purity with no discernible variation in cellular composition between isolated samples. Cell viability in the microvessel fraction was determined by dye-exclusion using trypan blue. No discernible damage to microvessel segments was apparent in any of the isolates. The parenchymal cell fraction consisted predominantly of neurons and glial cells with little or no vascular contamination. Before blotting, microvessel and microvessel-deleted brain samples were pelleted in a high speed microfuge, rediluted in 50 μl sucrose-free buffer, and sonicated in an icebath for 1 to 2 min. Samples were re-centrifuged and the supernatant collected for Western blotting. Protein concentration was determined spectrophotometrically at 595 nm using the method described by Bradford (1976).

Electrophoresis and Western blotting

Mouse microvessel or brain parenchymal cell proteins (10 μg/lane) were loaded onto 4–20% gradient Tris–glycine gels (Invitrogen, Carlsbad, CA), and separated by polyacrylamide gel electrophoresis (SDS-PAGE) run for 2 h at 150 V. Proteins were blotted onto either nitrocellulose or polyvinylidene difluoride (PVDF) membrane and incubated with a primary antibody diluted 1:1000, overnight at 4 °C. Duplicate blots were subsequently exposed for 1 h to the appropriate (antimouse or antirabbit) HRP-conjugated secondary antibody diluted 1:2000. Consistency of sample loading was assessed by staining blots with Coomassie blue, and reprobing with antibody to β-actin. Western blots for detection of MnSOD were exposed to Kodak X-Omat AR film (Rochester, NY). Blots for the detection of other proteins were exposed to Kodak BioMax ML film. Band densities were determined using a Bio-Rad GS-670 Imaging Densitometer (Hercules, CA).

Blood gas analysis for PaO_2

Arterial blood samples for PaO_2 determination (100 μl) were taken from 6-, 18-, and 24-month-old mice subjected to ambient