Research report

Localization of extracellular superoxide dismutase in adult mouse brain

Tim D. Oury a, J. Patrick Card b, Eric Klann b,c,*

a Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA 15261, USA
b Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA 15260, USA
c Center for Neural Basis of Cognition, University of Pittsburgh, Pittsburgh, PA 15260, USA

Accepted 7 September 1999

Abstract

Extracellular superoxide dismutase (EC-SOD) is one of three mammalian SOD isozymes. Although there is knowledge of the functional role of EC-SOD in arteries, little is known about the function of EC-SOD in other tissues, including the brain. As a first step toward improving our understanding of EC-SOD in the brain, we studied the localization of EC-SOD in the central nervous system of the adult mouse using immunohistochemistry. We detected EC-SOD staining in a subpopulation of neurons throughout the brain as well as in tanycytes in the mediobasal hypothalamus. Particularly prominent EC-SOD staining was observed in neurons of the hilar region of the hippocampus, the lateral habenular nucleus of the thalamus, and the suprachiasmatic nuclei of the hypothalamus. Substantial numbers of neurons were distributed throughout the striatum and cortex; the morphology and distribution of these cells was similar to neurons previously shown to contain the neuronal isoform of nitric oxide synthase. In contrast to other regions with prominent EC-SOD immunoreactivity, EC-SOD localization in tanycytes occurred in a region lacking a blood-brain barrier. The high levels of EC-SOD present in discrete populations of cells in these regions suggest that EC-SOD plays an important, specialized role in the physiology and/or pathology in the brain. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Superoxide; Nitric oxide; Hippocampus; Hypothalamus; Thalamus; Suprachiasmatic nucleus

1. Introduction

Extracellular superoxide dismutase (EC-SOD) is one of three mammalian SOD isozymes. In general, there is a small amount of EC-SOD in most tissues compared to the intracellular CuZn and Mn forms of SOD [12,20]. One exception to this general rule is that EC-SOD is the predominant isozyme in arteries, where it contributes up to 70% of the total SOD activity [19,21,23].

Nitric oxide is an important intercellular signaling molecule in many systems and plays a prominent role in modulating vascular tone by signaling smooth muscle relaxation [20]. Nitric oxide also is known to be very sensitive to superoxide-mediated degradation. The reaction of nitric oxide with superoxide proceeds at diffusion-limited rates to produce the toxic peroxynitrite anion [9,10].

One proposed function for the high levels of EC-SOD in the extracellular matrix of arteries is to prevent superoxide-mediated inactivation of nitric oxide [20]. In support of this idea immunolocalization studies have shown that EC-SOD is situated in the extracellular matrix around smooth muscle cells, the same matrix nitric oxide must traverse to signal smooth muscle relaxation [20,21].

The function of EC-SOD in other tissue matrices is less certain. The relatively low level of EC-SOD activity in most tissues has led to the speculation that EC-SOD is not a bulk scavenger of superoxide in these tissues [7]. However, EC-SOD contains a heparin-binding tail in the carboxyterminal region of each of its four identical subunits [22]. This heparin-binding domain may permit localization of relatively high concentrations of EC-SOD in extracellular matrices where scavenging of extracellular superoxide is of critical importance. For example, in the lung EC-SOD has been shown to specifically localize in association with type I collagen in alveolar septal tips [19]. Interestingly, type I collagen is extremely sensitive to superoxide-mediated degradation [6,15,16]. Thus, the localization of EC-SOD in association with type I collagen in the extracellular domain appears to have a specific and important function.

The heparin-binding domain of EC-SOD also is likely to be important for tight regulation of enzymatic activity. For example, the heparin-binding domain of EC-SOD is
sensitive to proteolysis [22]. Upon proteolysis, EC-SOD loses its affinity for heparin and is cleared from the tissue into the serum. This feature allows for precise proteolytic regulation of EC-SOD activity.

The activity of EC-SOD in the brain is very low compared to most other organs [13,17]. However, alterations in EC-SOD activity using transgenic and knockout mice elicit profound physiological and pathological effects in the brain. For example, EC-SOD transgenic mice expressing a five-fold increase in EC-SOD activity in the brain were found to be more sensitive to central nervous system (CNS) oxygen toxicity compared to wild-type mice [17]. The increased sensitivity to CNS oxygen toxicity appeared to be due to augmentation of nitric oxide-mediated vascular relaxation by EC-SOD (i.e., inhibition of the protection provided by oxygen-induced vasoconstriction). On the other hand, EC-SOD transgenic mice were found to be protected against vasogenic edema [18]. Thus, depending on the type of brain injury, elevated EC-SOD activity can either protect against or exacerbate the injury.

EC-SOD may also play a role in normal brain function. For example, it was shown that EC-SOD knockout mice that have no EC-SOD activity are impaired in tasks requiring hippocampal-dependent spatial learning and memory [11]. These studies suggest that although the overall level of EC-SOD in the brain is low, there may be specific brain regions such as the hippocampus in which localized concentrations of EC-SOD are necessary for normal learning and memory functions.

The studies described herein examine the immunohistochemical distribution of EC-SOD in brains of normal mice. The determination of the localization of EC-SOD in the brain should provide insight into the specific physiological and pathological functions of this enzyme.

2. Materials and methods

2.1. Animals

Five adult (C77BL/6 × C3H)F1 male mice weighing 24–26 g were used in this investigation. They were maintained in constant conditions (12-h light; light on at 0700 h) and had free access to food and water. Experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2. Antiserum

A rabbit polyclonal antiserum generated against a synthetic peptide corresponding to the first 19 amino acids of mouse EC-SOD and containing a cysteine residue at position 20 was used for the immunohistochemical localizations. Details regarding the preparation and specificity of this antiserum have been published elsewhere [5]. To further establish the specificity of staining in the present study we simultaneously processed sections from the same brain with (1) primary antibody, (2) pre-immune serum, and (3) with antibody in which anti-EC-SOD IgG was absorbed out using purified mouse IgG as previously described [19].

2.3. Tissue preparation and processing

Each animal was anesthetized deeply by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7 mg/kg) prior to sequential transcardial perfusion of physiological saline (30–50 ml) and paraformaldehyde–lysine–periodate (200–300 ml) fixative [14]. The solutions were infused at a controlled pressure using a peristaltic pump (Cole-Palmer). Upon completion of the perfusion the brain was removed, postfixed for 1 h, and cryoprotected overnight in a phosphate buffered sucrose solution, all at 4°C. It then was sectioned serially in the coronal plane at 35 μm/section through the rostrocaudal extent of forebrain and brainstem using a sliding microtome equipped with a freezing stage (Leitz Physitemp). Sections were collected sequentially in four bins of cryoprotectant [25] and stored at −20°C until processed for immunohistochemical localization of EC-SOD.

One bin of tissue from each brain (section frequency of 140 μm) was processed for localization of EC-SOD using the avidin–biotin modification of the immunoperoxidase procedure [8]. Cryoprotectant was removed from the tissue by four sequential washes in 0.1 M sodium phosphate buffer on a rocker table. Sections then were transferred to a primary antiserum diluted to a concentration of 1:1000 in 10 mM sodium phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. Following a 48-h incubation at 4°C the tissue was washed in three changes of 10 mM buffer over 45 min prior to and following a 1-h incubation in affinity purified biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The sections then were incubated in ABC complex using reagents from the Vector Elite Kit (Vector Laboratories) for 90 min at room temperature on a rotator, washed in 10 mM phosphate buffer, and then incubated in a 0.1 M Tris-saline buffer containing dianisobenzidine (DAB) for 10 min. The immunoperoxidase reaction product was visualized by adding 35 μl of 30% H2O2/100 ml to the DAB solution. The H2O2-catalyzed DAB reaction was monitored visually and terminated by repeated washes in 10 mM phosphate buffer. Sections then were mounted on gelatin coated slides, dried at room temperature, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped with Cytoseal60.

2.4. Tissue analysis

All sections were analyzed with a Zeiss Axioplan photomicroscope equipped with differential interference
optics. Images of immunoreactive cells were digitized using a DAGE video camera (MTI 3CCD) and an image analysis system (Simple32; C-Imaging Systems). Adobe Photoshop software was used to assemble Figs. 1 and 3 of this report and to adjust contrast and brightness of individual photomicrographs in each plate so that they were of uniform density. An absolute map of the distribution of immunoreactive cells in two coronal planes from one animal was produced using a Nikon Optiphot microscope equipped with a drawing tube. The maps of these three sections, produced with the 10× objective, were then digitized and assembled into a single plate using Adobe Illustrator software.

3. Results

3.1. Specificity of immunohistochemical staining

The EC-SOD polyclonal antiserum stained the same population of cells in all cases. These cells were concentrated differentially within circumscribed regions of the CNS and, with the exception of hypothalamic tanycytes, exhibited the morphology of neurons. Processing of sections with pre-immune serum or antibody that had been pre-absorbed on a column failed to produce staining of any population of cells. Fig. 1A through C demonstrate this specificity using one of the populations of CNS neurons that stained most heavily for EC-SOD, those found in the hilus of the hippocampus. Fig. 1A illustrates a low magnification photomicrograph through the caudal hippocampus and cortical mantle. Even at low magnification the densely staining populations of neurons in the hilus (boxed area of Fig. 1A and B) are readily apparent. Higher magnification illustrates the dense immunoreactivity typically observed in hilar neurons found between the blades of the dentate granule cell (gc) layer. Staining of these cells was not observed in tissue processed with either the pre-immune serum (data not shown) or pre-absorbed antiserum (Fig. 1C), both of which also failed to stain all other populations of cells identified with the primary antiserum.

3.2. Subcellular localization of EC-SOD immunoreactivity

At low to intermediate magnifications the EC-SOD staining appeared as a homogeneous staining of the cyto-

Fig. 1. Specificity of the antiserum and intracellular distribution of EC-SOD immunoreactivity is demonstrated. Panel A shows low magnification image of a coronal section through caudal hippocampus illustrating the dense concentration of EC-SOD immunoreactive neurons in the hilus (boxed area). The same region is shown at higher magnification in panels B and C, with panel B demonstrating the distribution and morphology of immunoreactive hilar neurons and panel C demonstrating the loss of specific staining resulting from pre-absorption of the antiserum. Panel D illustrates the intracellular distribution of EC-SOD immunoreactivity in hilar neurons. Note the granular nature of the immunoperoxidase localization. Marker bars for panels A through C are 100 μm. Abbreviations used in panels B and C: gc, granule cells.
plasm of immunoreactive neurons. No staining of cell nuclei or processes was observed. Higher magnification
analysis confirmed that staining was confined to the cytoplasm of neurons but revealed that it was granular (e.g.,
vesicular) rather than diffuse. This staining pattern is
demonstrated in Fig. 1D and E. These images are from the
same field shown in Fig. 1B but were obtained with a
100 × oil immersion objective. Note the paucity of staining
in the cell nuclei and the granular nature of the staining
in the cytoplasm of each cell. This subcellular localization
typified all immunoreactive neurons, irrespective of their
localization in the brain.

3.3. Distribution of immunoreactive neurons

The maps shown in Fig. 2 illustrate the distribution of
EC-SOD-immunoreactive neurons in two sections from
one of the experimental animals. The coronal planes se-
lected for illustration demonstrate the regions of mouse
CNS that contain the highest concentration of immunore-
active cells. It is important to note that the maps define the
absolute distribution of immunoreactive neurons in the
case subjected to analysis and are representative of the
distribution seen in the same areas of the other cases. However, no quantitative inferences should be made re-
garding the number of immunoreactive neurons across
cases. Sections 3.3.1, 3.3.2, 3.3.3, 3.3.4 and 3.3.5 describe
the morphology and distribution of immunoreactive neu-
rons in the different regions of the brain.

3.3.1. Cortex

EC-SOD immunoreactivity was observed in small neu-
rons throughout the cortical mantle. These cells were
approximately 10 μm in widest diameter, multipolar in
conformation, and were sparsely distributed through all
cortical laminae (Figs. 2 and 3A). No immunoreactivity
was observed in neurons exhibiting pyramidal cell mor-
phology. Immunopositive cells appeared to be more preva-
lent in frontal and temporal cortices and less prevalent in
parietal cortex. In addition, larger numbers of immunoreac-
tive cells were observed in the subiculum and the entorhi-
nal cortex.
3.3.2. Striatum

One of the largest concentrations of EC-SOD immunopositive neurons was found in the striatum (Fig. 2). These cells were distributed uniformly through the striatum and were continuous with immunoreactive cells of similar morphology and relative density in the subjacent nucleus accumbens and ventral pallidum. The cells were 10–15 μm in widest diameter with 3–5 small processes emanating from the somata (Fig. 3B). They exhibited a marked similarity to striatal neurons previously shown to contain nitric oxide synthase immunoreactivity (for a review, see Ref. [24]).

3.3.3. Hippocampus

As noted previously, the hilus contained one of the most prominent groups of EC-SOD immunoreactive neurons (Fig. 1A and B). These cells were typical of hilar neurons in that they exhibited oblong perikarya with a long axis of 15–20 μm and gave rise to approximately three primary dendrites. Granular EC-SOD immunoreactivity was concentrated densely in these neurons and could occasionally be followed into the proximal portions of primary dendrites (Fig. 1D). Immunoreactive neurons also were observed in other cytoarchitecturally distinct hippocampal subdivisions throughout its rostrocaudal extent. Most of these neurons exhibited a morphology and distribution consistent with interneurons. Occasionally, immunoreactivity was observed in neurons within the pyramidal cell layer, and a small number of these cells exhibited pyramidal cell morphology. Qualitative observations suggested that EC-SOD immunoreactive neurons were more prevalent in the CA3 region of Ammon’s horn compared to the CA1 region (compare Fig. 3C and D).

3.3.4. Diencephalon

The thalamus and the hypothalamus contained three of the most prominent EC-SOD immunoreactive populations of neurons in the neuraxis. Dense accumulations of immunopositive cells were observed in the lateral habenular nucleus of thalamus (Fig. 3E), the suprachiasmatic nuclei (SCN) (Fig. 3F), and the ventral tuberal area of hypothalamus (Fig. 3G). Within each of these regions the immunoreactive cells were concentrated differentially in a subset of the cells. In the habenula, immunoreactive neurons were restricted to the lateral nucleus and were enriched within neurons found in a small subfield at the ventral and medial portion of the nucleus (Fig. 3F). Immunoreactive neurons were not found in other thalamic nuclei. The SCN contained prominent accumulations of immunoreactive neurons that were restricted to the ventrolateral portion of the nuclei that receive retinal innervation (Fig. 3E). Neurons in this portion of the SCN also are distinguished by their neurochemical phenotype. In particular, large numbers of neurons that express vasoactive intestinal polypeptide, gastrin releasing peptide, and the neuronal isoform of nitric oxide synthase have been reported in this portion of the SCN [4].

The tuberal hypothalamus contained a particularly prominent population of EC-SOD immunoreactive cells (Fig. 3G). Interestingly, EC-SOD localization in this region was not confined to neurons. The predominant localization was observed within the specialized ependymal cells (tanycytes) whose tail processes extend between the lumen of the third ventricle and the capillary plexus and pial surface on the external surface of the brain [3]. It is important to note that the absence of the blood–brain barrier in this region provides the substrate for hypothalamic regulation of anterior pituitary function and that tail processes of tanycytes are thought to contribute to the reestablishment of the barrier function in areas abutting upon adjacent hypothalamic nuclei. EC-SOD immunoreactivity was dense through both the perikarya and processes of tanycytes, including the endfeet that terminate upon fenestrated capillaries in the median eminence (Fig. 3G). Occasional immunoreactive neurons also were observed in the arcuate nucleus of hypothalamus, which is traversed by tanycytic processes (Fig. 3G).

3.3.5. Other regions of the CNS

Only a small number of immunoreactive cells were present in the midbrain or hindbrain. In the cerebellum, scattered EC-SOD immunopositive cells were observed among the parallel fibers of the molecular layer. These small multipolar cells were few in number and sparsely distributed. Similar sparsity of staining was apparent in the brainstem with only occasional cells observed in the central tegmentum. No cell group or region of the brainstem exhibited preferential accumulation of immunoreactive cells.

4. Discussion

In this report, we have used immunohistochemical methods to show for the first time that EC-SOD is localized to neurons in the adult mouse brain. The most intense immunohistochemical staining was observed in discreet populations of neurons in the hippocampus, striatum, SCN, and the habenula. Interestingly, EC-SOD appeared to be localized primarily in intracellular vesicle-like structures (Fig. 1D and E). The intracellular localization of EC-SOD in these neuronal populations is in sharp contrast to the localization of EC-SOD in most other tissues where it is found in the extracellular matrix [19,21]. There are several possible explanations for this observation. First, the levels of EC-SOD present in the extracellular space may be too low for immunohistochemical detection. Second, the heparin-binding domain of EC-SOD is sensitive to proteolysis [22]. Thus, proteolysis might have occurred during either
the perfusion of the animal or the fixing of the tissue, resulting in a loss of extracellular immunoreactivity. Notably, EC-SOD previously has been detected intracellularly in extravillous trophoblasts in human placentas [1]. Interestingly, EC-SOD also has been observed intracellularly in villous trophoblasts of first and early second trimester placentas before being excreted into the extracellular matrix of the villi later in the second trimester (Bogges and Oury, unpublished observations). Thus, it is possible that EC-SOD is stored in secretory vesicles within neurons and excreted under conditions in which EC-SOD is needed extracellularly.

What is the function of EC-SOD in the brain? It is well known that superoxide reacts rapidly with nitric oxide to form the toxic peroxynitrite anion [9,10]. Therefore, it is possible that EC-SOD prevents the formation of peroxynitrite and permits the actions of nitric oxide in subsets of neurons in the SCN and the striatum.

Two areas of the brain exhibited particularly prominent EC-SOD immunoreactivity. One area is the tuberal hypothalamus (Fig. 3G). In contrast to other brain regions where EC-SOD was localized predominantly to neurons, EC-SOD localization in this region was almost exclusively localized within tanyctyes. The absence of the blood–brain barrier in this region provides the substrate for hypothalamic regulation of anterior pituitary function. Because this is an area of relatively high blood flow, EC-SOD may enhance nitric oxide-dependent vasodilation to maintain the high blood flow in this area. In addition, the lack of a blood–brain barrier in this area may also make this region more sensitive to inflammatory reactions. Thus, EC-SOD in this region may serve to protect against any increased superoxide that is produced due to the absence of the blood–brain barrier.

The hilus of the hippocampus also exhibited prominent EC-SOD immunoreactivity (Fig. 1A and B), primarily in neurons with a morphology and distribution consistent with interneurons. Aspiny fusiform hilar interneurons in this area receive medial entorinal input and have long septotemporal projections that are confined to the dentate gyrus [2]. Spiny hilar interneurons project to the molecular layer of the dentate gyrus, as well as to pyramidal neurons in areas CA3 and CA1 [2]. The spiny hilar cell projections are less extensive than the aspiny fusiform hiliar cell projections. Because EC-SOD knockout mice have deficient hippocampal-dependent spatial learning and memory [11], it is possible that EC-SOD in aspiny fusiform and/or spiny hilar interneurons plays a critical role in information processing in the hippocampus.

Collectively, our findings show that EC-SOD is localized in discreet populations of cells, mainly neurons, in the adult mouse brain. This discrete localization suggests specialized functions for EC-SOD under normal physiological and pathological conditions in the brain.

Acknowledgements

We would like to thank Jen-Shew Yen and Lisa M. Schaefer for technical assistance, and Dr. Edda Thielis for thoughtfull comments on the manuscript. This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-34007 (E.K.) and seed awards from the National Parkinson’s Foundation Center for Excellence at the University of Pittsburgh (T.D.O. and J.P.C.).

References


