The immunocytochemical demonstration of copper–zinc superoxide dismutase in the brain

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Summary

Copper-zinc superoxide dismutase (CuZn-SOD) has been localized in formalin-fixed, paraffin-embedded sections of both canine and rat brains. Staining with an immunoenzyme bridge sequence revealed CuZn-SOD in all regions of the brains examined. Specific sites of localization included cerebral cortical pyramidal cells, cerebellar Purkinje cells, neurons in 'subcortical nuclei', and oligodendrocytes throughout the brain. Similar sites of CuZn-SOD localization were identified in both species. These results are compared with reports by various investigators of SOD bioactivity in the brain.

Introduction

Superoxide, a free radical generated during the monovalent metabolic reduction of oxygen, is toxic to cells in which it is produced. The enzyme superoxide dismutase (SOD; EC 1.15.1.1) scavenges this radical and keeps its concentration at a homeostatically low level in cells and tissues. SOD has been shown to inhibit lipid oxidation (Fridovich, 1975) and to prevent damage to DNA by oxygen radicals (Uchigata et al., 1982). It is also capable of diminishing cell damage caused by the chemical diabetogenic agents alloxan and streptozotocin in pancreatic beta cells (Grankvist et al., 1979; Fischer & Hamburger, 1980; Gandy et al., 1982; Uchigata et al., 1982). Turrens et al. (1984) have shown that SOD increases the survival time of rats continuously exposed to a 100% oxygen environment, a condition known to cause severe damage to the nervous system (Balentine, 1982). Majewska et al. (1978) have reported that the oxidation of phospholipids by oxygen-derived free radicals in the guinea pig brain is increased by ischaemia. It seems clear, then, that the CNS is highly susceptible to damage by free radicals generated under a variety of conditions of high and low oxygen tension. For this reason, the specific accommodation of superoxide by various cell types in the brain is of interest to neurobiologists.

Three types of SOD are responsible for producing the activity attributed to this enzyme: a cytoplasmic copper–zinc SOD (CuZn-SOD), a mitochondrial, manganese SOD (Weisiger & Fridovich, 1973) and an extracellular SOD of high molecular weight (Marklund et al., 1982a). The specific localization of CuZn-SOD has been reported recently for a variety of canine tissues (Thaete et al., 1983) including some neurons. Various parts of the brain are known from biochemical studies to exhibit differing levels of SOD activity (Thomas et al., 1976; Van Balgooy & Roberts, 1979; Ledig et al., 1982). The immunocytochemical approach would extend knowledge of the location of the enzyme to the cellular level. Correlation of the presence of the enzyme in specific neuronal types with the known environment and function of these neurons could then provide new insight into the physiologic significance of the enzyme. This study undertook to determine the cellular distribution of CuZn-SOD in the CNS of the dog and the rat.

Methods

Tissues

Brains were obtained from anaesthetized adult dogs and both 10-day-old and adult Wistar rats, which were housed and treated according to the Medical University of South Carolina's guide for the care and use of laboratory animals. All animals were provided with food and water ad libitum. Five adult dogs were anaesthetized by intravenous injection of sodium pentobarbital (25 mg kg⁻¹) and sacrificed at the time the brain was removed. Eleven adult rats and six rat pups (10 days old) were sacrificed by cervical dislocation. The brain of each animal was removed and slices (1–2 mm thick) of various parts (dog) or of the whole brain (rat) were fixed in 4% formalin buffered with...
2% calcium acetate (pH 6.5). After a 2 h fixation period the brain slices were rinsed for 2 h in running water, then dehydrated through a graded series of ethyl alcohols and embedded in paraffin.

Immunolocalization

CuZn-SOD was localized on 5-µm sections using a modified immunoglobulin peroxidase bridge sequence (Mason et al., 1969) employing biotinylated goat anti-rabbit IgG (GAR) and an avidin–biotin–peroxidase complex (ABC) (Hsu et al., 1981). Purified canine CuZn-SOD was obtained from Sigma Chem. Co. (St. Louis, MO) and rat CuZn-SOD was purified according to the method of Crapo & McCord (1976). The enzyme preparation was assayed for purity by polyacrylamide gel electrophoresis (Beauchamp & Fridovich, 1971) and used to develop antisera in rabbits as described previously (Crouch et al., 1981; Thaete et al., 1983). Radioimmune assays indicated that 1 g CuZn-SOD could be precipitated with 1.0 ml of antiserum diluted 1:2000 for both dog and rat.

Deparaffinization and rehydration of the sections were followed by a 10-min treatment in H$_2$O$_2$ to inactivate endogenous peroxidase. This treatment is capable of eliminating the enzymatic activity of CuZn-SOD (Hodgson & Fridovich, 1975) but does not diminish the immunoreactivity of the molecule as measured by radioimmunoassay (R. Crouch, unpublished observation). Sections were then washed for 5 min in 0.1 M phosphate-buffered saline (PBS), pH 7.2, followed by a 15-min treatment in normal goat serum (diluted 1:5) to block nonspecific binding. Canine brain sections were then treated with rabbit anti-canine CuZn-SOD (1:500) and rat sections were treated with rabbit anti-rat CuZn-SOD (1:800), both overnight at 4°C. A 10-min rinse in three changes of PBS followed this and all subsequent steps. The remaining steps in the immunostaining sequence were as follows: biotinylated GAR, 1 h at room temperature; ABC (Vector Labs, Burlingame, CA), 1 h at room temperature; and development in substrate medium containing 0.03% 3,3'-diaminobenzidine and 0.006% H$_2$O$_2$ in PBS, 15 min at room temperature. Sections were then dehydrated and mounted in Permount without counterstaining.

As controls for the immunostaining procedure, brain sections adjacent to those which were incubated with primary rabbit antisera to CuZn-SOD were treated with either pre-immune serum from the same rabbit that generated the antiserum, or with rabbit anti-CuZn-SOD which had first been adsorbed with 100 µg ml$^{-1}$ (canine) or 200 µg ml$^{-1}$ (rat) of very highly purified CuZn-SOD.

To identify glial cells, some sections were stained in an immunoperoxidase bridge sequence using anti-glial fibrillary acidic protein (GFAP, Dako Corp., Santa Barbara, CA) as the primary antisera. This procedure stains astrocytes and ependymal cells but not other glial elements. Oligodendrocytes were identified using anti-galactocerebroside (GO) (kindly provided by G. Sobue and D. Pleasure, Children’s Hospital, Philadelphia, PA) which binds to the myelin surface of these cells (Raff et al., 1978, 1983; Sobue & Pleasure, 1984).

Results

Immunoreactive CuZn-SOD was consistently localized in both neuronal and glial elements of dog and rat brains.

Neurons varied in immunostaining for SOD, and in order to assess the relative reactivity for diverse neuronal groups, the investigation must be extended. The widespread distribution of SOD-containing neurons, however, can be documented by listing several immunopositive sites. Cortical pyramidal cell bodies and many of their processes, as well as non-pyramidal cells, were stained in the cerebrum (Fig. 1). Neurons in a variety of subcortical nuclei also exhibited clear immunostaining. Neurons in the pons disclosed diffuse cytologic staining extending into the neuronal processes (Fig. 2). In the cerebellum, Purkinje cells and their dendrites were the most prominently stained type of cell (Figs 3, 4); stellate cell bodies also appeared to contain the enzyme.

Oligodendrocytes were judged to be the only glial cell that stained consistently for CuZn-SOD. Astrocytes stained well with anti-GFAP (Fig. 5). However, in adjacent sections stained for CuZn-SOD the astrocytes appeared unreactive (cf. Figs 5, 6). More rounded cells were identified as oligodendrocytes by their immunoreactivity for galactocerebroside and these corresponded in size, shape and distribution with the CuZn-SOD-positive cells (cf. Figs 6, 7). These glial cells stained for CuZn-SOD in all regions of the brain examined.

In both dog and rat the same cell types were well stained for CuZn-SOD. Brains from rats of different ages showed immunostaining for CuZn-SOD in the...