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# Enzymatic Protection Against Peroxidative Damage in Isolated Brain Capillaries

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**Abstract:** The content of polyunsaturated fatty acids, the activities of superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase, and the concentration of reduced glutathione were measured in cerebral microvessels isolated from rat brain. Polyunsaturated fatty acids, mainly arachidonic, linoleic, and docosahexaenoic acids, accounted for 32% of total fatty acids in cerebral microvessels. Whereas total SOD activity in the microvessels was slightly lower than that found in cerebrum and cerebellum, glutathione peroxidase and glutathione reductase activities were twice as high and catalase activity was four times higher. Glutathione peroxidase in microvessels is active on both hydrogen peroxide and cumen hydroperoxide, and it is strongly inhibited by mercaptosuccinate. After several hours of preparation, the concentration of reduced glutathione in isolated microvessels was 0.7 μmol/mg of protein, which corresponds to a concentration of ~3.5 m*M*. Our results indicate that the blood–brain barrier contains large amounts of peroxide-detoxifying enzymes, which may act, in vivo, to protect its highly polyunsaturated membranes against oxidative alterations. **Key Words:** Blood–brain barrier—Polyunsaturated fatty acids—Peroxides—Glutathione peroxidase—Superoxide dismutase—Catalase. **Tayarani I. et al.** Enzymatic protection against peroxidative damage in isolated brain capillaries. *J. Neurochem.* **48,** 1399–1402 (1987).

The blood–brain barrier (BBB), which is composed of capillary endothelial cells, restricts and regulates the movement of ions and substrates between the plasma and the CNS. Although the oxygen demand is especially high in the brain (Rapoport, 1976), the uncontrolled activation of oxygen leads to lipid peroxidation and tissue damage (Tappel, 1973). Under normal conditions, part of the oxygen that is metabolized in the mitochondria undergoes univalent reduction, leading to superoxide and hydrogen peroxide formation (Forman and Boveris, 1982; McCord, 1983). Another source of oxygen free radicals is the ischemic activation of xanthine dehydrogenase to xanthine oxidase, which, upon postischemic reperfusion, catalyzes the conversion of hypoxanthine to xanthine and then to uric acid with stoichiometric generation of superoxide. The resulting burst in superoxide production leads to necrosis in many peripheral tissues (McCord, 1985). Although the brain exhibits little xanthine dehydrogenase/oxidase activity, the presence of xanthine oxidase in brain microvessels (Betz, 1985) suggests that superoxide may cause peroxidative damage to the BBB.

Peroxidation of polyunsaturated fatty acids is known to alter membrane fluidity (Dobretsov et al., 1977), enzyme activities (Esterbauer, 1985), and transmembrane ion fluxes (Augustin et al., 1979). Chan et al. (1984) have demonstrated that free radicals affect the permeability of the BBB to sodium and potassium and induce the release of free fatty acids. That $Na^+,K^+$-ATPase from cerebral microvessels is strongly affected by high concentrations of either superoxide (Lo and Betz, 1986) or lipid peroxides (Koide et al., 1986) further supports the hypothesis that free radicals induce alterations in the permeability to sodium and potassium.

Various protective enzymes help to maintain low steady-state concentrations of oxidizing species in the cell (Rotilio and Bannister, 1984). As glutathione peroxidase, catalase, and superoxide dismutase (SOD) provide a major detoxication pathway for peroxides in the brain (Brannan et al., 1980; Mavelli et al., 1982;

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*Abbreviations used:* BBB, blood–brain barrier; SOD, superoxide dismutase.

Ono and Okada, 1984), we measured the activities of these enzymes in isolated rat brain microvessels and compared them with activities in cerebrum and cerebellum.

## MATERIALS AND METHODS

Adult male Sprague-Dawley rats weighing 300–350 g and kept under diurnal light conditions were used in these experiments. Rat chow from U.A.R. (France) and water were available ad libitum. The animals were anesthetized by diethylether inhalation. The left heart was perfused with 50 ml of ice-cold physiological saline containing 7% heparin. The brain was quickly removed, and all subsequent steps were carried out at 4°C. Cortical shells, free of choroid plexus and of any visible superficial blood vessels or ependyma, were placed in a buffered aqueous solution containing 135 m*M* NaCl, 4 m*M* KCl, 3.2 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 15 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5 m*M* glucose, and 1% bovine serum albumin, pH 7.4 (prep buffer).

Brain capillaries were isolated according to the method of Goldstein et al. (1975) with a minor modification. In brief, a 4:1 (wt/vol) homogenate of cortical shells was made at 400 rpm and pelleted by centrifugation at 1,000 *g* for 10 min from an ~10% (wt/vol) suspension. The pellet was resuspended (12:1 wt/vol) in prep buffer, free of bovine serum albumin and containing 17.5% dextran (MW, 70,000), and then centrifuged at 4,000 *g* for 10 min. Pellets were resuspended in prep buffer and gently teased through a nylon mesh with 118-μm pores. The filtrate was passed through glass bead (diameter, 0.25 mm) columns (1 × 1 cm) and rinsed with an excess volume of prep buffer. The glass beads were then placed in a large volume of prep buffer, and the floating capillary segments were collected by centrifugation at 1,000 *g* for 5 min. Capillary pellets were then resuspended and washed twice, in 50 ml of a 0.9% NaCl solution, by centrifugation at 1,000 *g* for 5 min. The final capillary pellet was resuspended in 1–2 ml of 50 m*M* Tris-HCl buffer, pH 7.6, containing 0.1 m*M* EDTA and 0.1% Lubrol (incubation buffer). The suspension was then sonicated for 30 s, followed by freezing, thawing, and resonication. The resulting homogenate was centrifuged at 3,000 *g* for 10 min. The supernatant was removed and used for measurements of enzyme activities. Aliquots were taken for protein content determination using the Biorad dye-binding assay. The pellet was used for extraction of total lipids (Radin, 1981), acidic transmethylation of total fatty acids (Luddy et al., 1959), and analysis of the resulting methyl esters by gas chromatography, using a 25-m-long fused silica column impregnated with Carbowax 20 M. Hepatadecanoic acid was used as an internal standard.

Homogenates of total cerebral cortex and cerebellum in incubation buffer were made during microvessel preparation, and treated in the same fashion as microvessel samples for all measurements.

The reduced glutathione content in a supernatant obtained from microvessel homogenates in 5% sulfosalicylic acid was determined by C18 reverse-phase HPLC of the monobromobimane adduct with fluorimetric detection, as described by Anderson (1985), with cysteine used as the external standard.

### Enzyme activity measurements

Glutathione peroxidase activity was measured by a modification of the method of Paglia and Valentine (1967): Measurements were done at pH 7.6 and 37°C and in the presence of 0.5 m*M* GSH. The assay mixture in incubation buffer consisted of 0.12 m*M* NADPH, 0.5 m*M* GSH, 1 unit/ml of glutathione reductase, and 0.2 m*M* of either cumene hydroperoxide or hydrogen peroxide (initial concentrations). NADPH disappearance was monitored at a wavelength of 340 nm.

Glutathione reductase activity was measured in incubation buffer at pH 7.4 and 37°C by monitoring NADPH disappearance at 0.2 m*M* glutathione disulfide and 0.12 m*M* NADPH (initial concentrations).

Catalase activity was measured at 37°C in incubation buffer at pH 7.4 using 10 m*M* hydrogen peroxide (initial concentration) as substrate (Aebi, 1984). The consumption of $H_2O_2$ was recorded at an absorption wavelength of 240 nm.

SOD activity was measured at 25°C in 50 m*M* phosphate buffer, pH 7.8, containing 0.1 m*M* EDTA. The reaction mixture consisted of 0.01 m*M* ferricytochrome C, 0.05 m*M* xanthine, and the amount of xanthine oxidase needed to reduce ferricytochrome C at a rate of 0.025 absorbance unit/min at 550 nm as described by Flohé and Otting (1984). SOD activity was expressed in terms of units/mg of protein, where 1 unit is defined as that amount of enzyme that inhibits the rate of cytochrome C reduction by 50% under the above conditions. Corrections for nonenzymatic activities were made in all measurements.

## RESULTS

The fatty acid composition of total lipids extracted from rat brain microvessels is shown in Table 1. Arachidonate, docosahexaenoate, and linoleate were the major polyunsaturated fatty acids. Polyunsaturated, monounsaturated, and saturated fatty acids accounted, respectively, for 32, 24, and 44% of total fatty acids in brain microvessels.

Glutathione peroxidase, glutathione reductase, SOD, and catalase activities were found in isolated microvessels. The activities of these enzymes, with the exception of SOD, were significantly higher in mi-

**TABLE 1.** *Fatty acid composition of total lipids extracted from rat brain capillaries*

| Fatty acid | % of total fatty acids |
|---|---|
| 16:0 | 14.8 ± 0.6 |
| 18:0 | 24.8 ± 1.2 |
| 18:1 n-7 | 4.4 ± 0.6 |
| 18:1 n-9 | 16.3 ± 1.9 |
| 18:2 n-6 | 6.6 ± 1.6 |
| 20:0 | 2.3 ± 0.7 |
| 20:1 n-9 | 1.4 ± 0.5 |
| 20:4 n-6 | 15.5 ± 1.3 |
| 22:0 | 2.3 ± 0.4 |
| 22:4 n-6 | 1.1 ± 0.5 |
| 22:5 n-6 | 1.5 ± 0.4 |
| 22:6 n-3 | 7.2 ± 0.9 |
| 24:1 | 1.8 ± 0.8 |

Results are the averages ± SD of five different determinations. Fatty acids were determined as the corresponding methyl esters by fused silica-capillary gas chromatography as described in Materials and Methods.

crovessels than in total homogenates of either cerebrum or cerebellum (Table 2). This also appears to be a characteristic of endothelial cells of cavernous bodies, which, in addition, have a considerable capacity for producing NADPH for the regeneration of GSH (Dobrina and Rossi, 1983). An efficient system of hydrogen peroxide degradation may thus be a general feature of endothelial cells in most vessels. Our method of SOD assay did not distinguish between Cu/Zn-SOD and Mn-SOD. As the glutathione peroxidase activity measured with hydrogen peroxide was roughly equivalent to that measured with cumene hydroperoxide, glutathione activity in cerebral microvessels may be due essentially to the selenium-dependent enzyme. This conclusion is reinforced by the complete inhibition observed at 0.1 m*M* with the selenium-specific inhibitor mercaptosuccinate for either substrate (Chaudière et al., 1984). The activities of glutathione peroxidase and glutathione reductase in microvessels were approximately twice those observed in cerebrum and cerebellum homogenates, whereas catalase activity was four times higher in cerebral capillaries than in the brain. By contrast, the activity of SOD in brain microvessels was slightly lower than in the cerebrum or cerebellum samples. The glutathione concentration in isolated cerebral microvessels was 0.71 ± 0.15 μmol/mg of capillary protein, equivalent to a concentration of ~3.5 m*M* based on a 20% protein content. Our current figure for the glutathione concentration in total brain homogenate is generally close to 2.5 m*M* (data not shown). Hence, the glutathione concentration, after several hours of preparation, remained higher in isolated brain capillaries than in the brain.

## DISCUSSION

Our results demonstrate that the activities of glutathione peroxidase, glutathione reductase, and catalase are higher in brain microvessels than in either cerebrum or cerebellum, whereas total SOD activities are similar in the three tissues. It should be noted that the brain homogenates were not myelin free. Thus, the ratio of glutathione peroxidase to SOD activity is higher in microvessels than in the cerebrum or the cerebellum homogenates. Such high enzymatic activities found in cerebral microvessels suggest that endothelial cells require as much, if not more, protection against peroxidative damage than do other brain cells. An important consequence of our results is that an adequate amount of selenium in the diet is required to protect brain capillaries. The high content of highly oxidizable polyunsaturated fatty acids in brain capillaries, especially 20:4 and 22:6, suggests that lipid peroxidation is easily initiated in this tissue. It should also be noted that the linoleic acid content of brain microvessels found in our experiments is twice that reported in neurons and oligodendrocytes and 13 and 22 times higher than in synaptosomes and myelin, respectively (Bourre et al., 1984).

SOD and glutathione peroxidase are essential for cellular defense against peroxidative damage (Wendel, 1980; Rotilio and Bannister, 1984). Yusa et al. (1984) reported that increased brain SOD and catalase activities protect the CNS against oxygen-induced alterations. SOD, catalase, and glutathione peroxidase constitute a group of mutually supportive enzymes. Glutathione peroxidase protects Cu/Zn-SOD against inactivation by hydrogen peroxide (Sinet and Garber, 1981; Blech and Borders, 1983). Under physiological conditions, catalase is located almost exclusively in peroxisomes (Gaunt and De Duve, 1976); therefore, hydrogen peroxide produced by the dismutation of superoxide is degraded by glutathione peroxidase. Whether catalase can afford an adequate protection at high concentrations of hydrogen peroxide remains to be demonstrated. Whereas SOD and catalase do not require coenzymes, glutathione peroxidase utilizes glutathione to reduce hydrogen peroxide to water and organic hydroperoxides to nontoxic alcohols. As the concentration of reduced glutathione within the cell

**TABLE 2.** *Comparison of specific activities in homogenates of rat cerebrum, cerebellum, and brain microvessels*

| Tissue | Enzyme | | | |
|---|---|---|---|---|
| | Glutathione peroxidase[a] | Glutathione reductase[b] | Catalase[c] | SOD[d] |
| Microvessels | 47.6 ± 5.3 | 48.4 ± 1.3 | 4.8 ± 0.3 | 48.0 ± 5.0 |
| Cerebrum | 24.0 ± 0.9 | 25.0 ± 2.9 | 1.3 ± 0.1 | 58.6 ± 1.6 |
| Cerebellum | 24.0 ± 0.8 | 23.7 ± 2.2 | 1.6 ± 0.2 | 57.7 ± 0.8 |

Results are the averages ± SD of four different determinations.

[a] In nmol of hydrogen peroxide reduced/mg of protein/min; at 0.5 m*M* GSH, pH 7.6, and 37°C.

[b] In nmol of NADPH oxidized/mg of protein/min; at 0.2 m*M* glutathione disulfide, pH 7.6, and 37°C.

[c] In μmol of hydrogen peroxide consumed/mg of protein/min; at 10 m*M* hydrogen peroxide, pH 7.4, and 37°C.

[d] In units/mg of protein, as described in Materials and Methods.

decreases, hydroperoxides can no longer be reduced by glutathione peroxidase (Smith et al., 1983). The observation of substantial amounts of GSH in cerebral microvessels after several hours of preparation suggests that glutathione is efficiently regenerated.

The fatty acid profile in this study is in agreement with previously reported results (Selivonchick and Roots, 1977; Matheson et al., 1980; Brown et al., 1984). If the polyunsaturated fatty acids of brain microvessels contribute to the high selectivity of the BBB, then they would help to protect the CNS against the toxic effects of peripheral molecules, but at the same time, they would enhance the susceptibility of the BBB to lipid peroxidation.

Our results suggest that peroxide-detoxifying enzymes in cerebral microvessels play an important role in protecting the functional integrity of the BBB, which in turn protects that of the brain.

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