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STATUS OF ANTIOXIDANTS IN BRAIN MICROVESSELS OF MONKEY AND RAT*

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Levels of certain antioxidants namely reduced glutathione (GSH), ascorbic acid (Vit C), α-tocopherol (Vit E) and antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were compared in monkey and rat brain microvessels which constitute the blood-brain barrier (BBB). The BBB of both the species contains appreciable amounts of the antioxidants to protect against oxidative damage. The level of protection in rat seems to be more efficient than monkey since rat microvessels contain higher concentrations of some of the bio-antioxidants. The comparative status of enzymatic and non-enzymatic protective system against oxidation in the brain microvessels has been discussed.

KEY WORDS: Brain microvessels, monkey, rat, antioxidants and antioxidative enzymes.

INTRODUCTION

The susceptibility of a given organ or organ system to oxidative stress is determined by the overall balance between the factors that exert oxidative stress and those that exhibit antioxidant capability. A number of antioxidant defence systems is present in the cell, which helps to protect it from the deleterious effects of oxidative stress. The most important chain breaking antioxidant inhibitor of lipid peroxidation in human is alpha-tocopherol.[1] Such antioxidants inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. As a major water soluble antioxidant, vitamin C is capable of maintaining sulfhydryl compounds in a reduced state, particularly in several redox reactions.[2,3] Vitamin C and reduced glutathione (GSH) may also be involved in the regeneration of vitamin E.[4,5] Among enzymatic antioxidants superoxide dismutase specifically scavenges superoxide radicals which is a major agent responsible for the generation of an array of reactive oxygen species.[6] Catalase and glutathione peroxidase are known scavengers of H_2O_2 and lipid hydroperoxide.[6]

Brain microvessels constitute the blood-brain barrier (BBB) which protects the brain from all kinds of damage including oxidative damage, as microvessels have been reported to contain antioxidative enzymes. The present communication deals with studies of enzymatic and non-enzymatic antioxidants in the brain microvessels of a non-human primate (rhesus monkey) and a rodent (rat), since there are striking differences in the sensitivity of these two species to chemical exposure. Monkey lungs were found to be more susceptible to oxidative injury produced by ozone as

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compared to rats[7] and primate brain is also more susceptible to Parkinsonian syndrome produced by MPTP toxicity because of less monoamine oxidase activity in monkey brain microvessels.[8] Also, a remarkable species difference in the activities of different antioxidative enzymes in anatomic sub compartments of lung has been observed.[9] It is, moreover, of interest to know whether there is a species difference or not as far as the distribution of these antioxidants in brain microvessels is concerned. The studies gain further importance in the light of our recent report showing the possible involvement of free radicals in hypertonic saline induced BBB disruption.[10]

MATERIALS AND METHODS

Animals

Rhesus monkeys (*Macaca mullata*), 5–7 kg and Sprague Dawley rats (200–225 g) were used in the study. The animals were maintained under standard approved husbandry conditions during the studies. The animals were perfused with chilled normal saline under ketamine anaesthesia. Brains were dissected out in the cold and were cleaned of superficial vessels. The cerebral cortices were processed for the isolation of microvessels at 0–40°C by the procedure of Palmer *et al.*[11] Briefly, after mincing, the brain was homogenized (10%, w/v) in 0.25 M sucrose, 5 mM EDTA and 50 mM Tris-HCl (pH 7.2), by passing it through nylon mesh of 600 μm and 135 μm respectively. The procedure was repeated thrice with each mesh. The homogenate (filtrate) was centrifuged at 1500 g for 10 minutes and the pellet thus obtained was loaded on discontinuous sucrose gradient having 1.0, 1.3, 1.5 and 1.8 M sucrose. Samples were centrifuged at 60,000 g for 60 minutes and pellet was suspended in 150 mM KCl and used immediately for biochemical estimation or stored at −70°C. The microvessel preparation was found to be quite pure in both the species as judged by several fold enrichment in alkaline phosphatase activity, in monkey (10.25 μmol product formed/mg/hr vs 1.54 μmol product formed/mg/hr) and rat (11.99 μmol product formed/mg/hr vs 1.23 μmol product formed/mg/hr) preparation respectively compared to whole brain activity. Phase contrast microscopy further confirmed the purity of microvessels fraction of both the species.

In case of rats, 6 brains were pooled to make one sample and there were 5 samples for each analysis. Five monkey brains were processed separately for biochemical assays.

Unnecessary exposure to light, air, heat, alkaline conditions and metal ions contamination was avoided. Glassware used was previously soaked in 10% nitric acid solution, washed thoroughly with glass distilled water and rinsed before use with redistilled ethanol.

Enzyme Assays

For enzyme assays, microvessel preparations were subjected to freezing/thawing followed by mechanical homogenization in cold.

Superoxide Dismutase (SOD, EC 1.15.1.2)

The enzyme activity was assayed by the method of Kakkar *et al.*[12] The inhibition of colored formazan complex formation resulting from reduction of O_2 with nitroblue tetiazolium (NBT) in the presence of phenazine methosulfate (PMS) and NADH was measured. One unit was defined as the amount of enzyme that inhibited the rate of reaction under the given conditions by 50%.

Glutathione Peroxidase (GPx, EC 1.11.1.9)

The assay of GPx was done by following the formation of oxidized glutathione in presence of reduced glutathione and tert-butylhydroperoxide as substrates.[13] This procedure assays total (Se and non-Se) GPx activity.

Catalase (CAT, EC 1.11.1.6)

The activity of CAT was estimated at 37°C in phosphate buffer, pH 7.2, using 10 mM H_2O_2, as substrate.[14] The disappearance of hydrogen peroxide was recorded at an absorption wavelength of 240 nm. The activity was expressed as units/mg protein.

Glutathione Reductase (GR, 1.6.4.2)

It was assayed by monitoring NADPH oxidation in presence of oxidised glutathione.[15]

Other Antioxidants

Glutathione (GSH) The supernatant obtained following protein precipitation by meta-phosphoric acid was estimated fluorometrically for GSH by orthophthaldialdehyde method of Cohn and Lyle.[16]

Ascorbic acid It was measured spectrophotometrically in the form of a colored complex formed with dinitiophenylhydrazine by the method of Rae.[17]

Vitamin E Vit E was estimated fluorometrically after saponification and extraction with hexane by the procedure of Desai.[18]

The recovery of the added standards was found to be in the range of 96 ± 2%, that showed no appreciable losses of nonenzymatic antioxidants during processing of the tissue. The methods used for various biochemical analyses were found to be reproducible both within batch and day to day experiments.

Protein was measured by the procedure of Lowry *et al.*,[19] using serum albumin as standard.

Statistical Analysis

Data obtained were analyzed for significance of difference between the two species by Student's 't' test. p values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

The direct analysis of reactive oxygen metabolites in a biological material is often difficult because of their intrinsic reactivity. Much of the evidence linking their production with physiological and pathophysiological events has come from the studies of the disposition of the host defense mechanisms themselves. In the present study, significant amounts of enzymatic and nonenzymatic antioxidants were observed in monkey brain microvessels. In the rat brain microvessels also comparative values were obtained. As far as the ratio of activity of free radical scavenging enzymes in microvessels as compared to whole brain is concerned (data not presented) the activity of GPx and GR were found to be almost double in microvessels in both the species. Although brain is known to have very little CAT activity compared to other organs, its microvessels fraction had approximately 3.5 fold higher CAT activity in comparison to whole brain which also appears to be very low and is consistent with our earlier report.[10] The activity of SOD was almost similar to little lower in microvessels of both the species compared to their whole brain values. An enrichment of approximately 1.5 fold in GSH and VIT E concentrations was also observed in microvessels of both the species. The activities of antioxidant enzymes SOD, CAT, GPx (total Se and non-Se) and GR were significantly lower in the monkey brain microvessels compared to the rat (Figure 1). Plopper *et al.*,[7] have also reported lower levels of SOD and GPx in lung sub compartments of primate

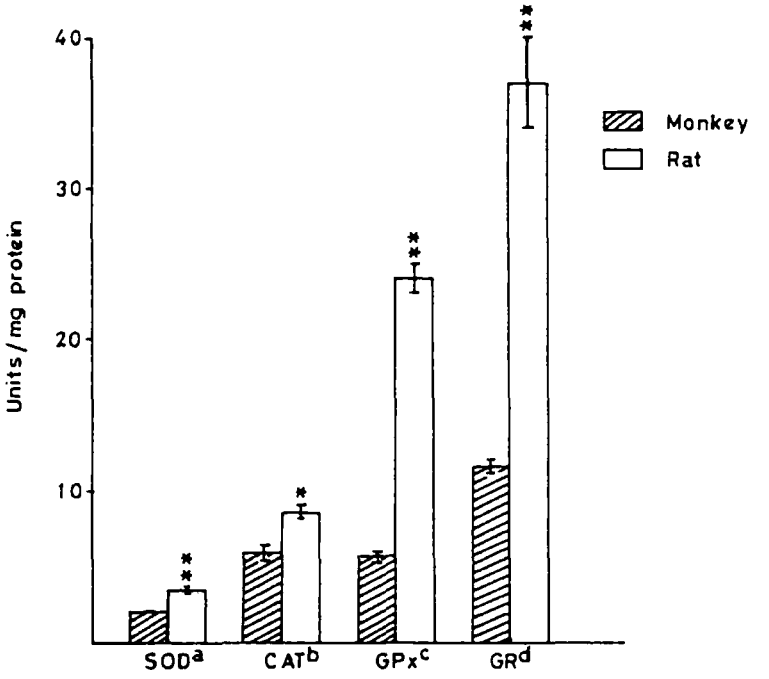

FIGURE 1 Free radical scavenging enzymes in monkey and rat brain microvessels $^{*}p < 0.005$; $^{**}p < 0.001$, [a]Units/mg of protein, [b]μmol of hydrogen peroxide utilized/mg of protein/min at 37°C, [c]nmol of oxidised glutathione formed/mg protein/min at 37°C, [d]nmol of NADPH oxidised/mg of protein/min at 37°C.

TABLE 1
Basal levels of nonenzymatic antioxidants in monkey and rat brain microvessels

Antioxidant	Monkey	Rat
GSH[a]	1.89 ± 0.11	1.05 ± 0.10
Vitamin E[a]	1.25 ± 0.08	1.52 ± 0.10
Vitamin C[a]	188.34 ± 9.17	162.56 ± 16.30

Values are Mean ± SE of five samples. Monkey brains were analyzed separately; however, six rat brains were pooled to make one sample. a μg/mg protein

as compared to rat lung showing higher sensitivity of primate to chemical exposure.

Vitamin E, was found to be present in the microvessels of both the species. In endothelial cell membrane, it serves as a hemostatic factor helping to prevent microvascular leakage and permeability defect[20] and is found in substantial amounts in other endothelial cells as well.[21] Ascorbic acid and reduced glutathione, which are known to regenerate Vit E in biological system apart from participating in many redox reactions and free radical scavenging,[2-5] were found to be present in substantial amounts in both the species (Table 1). The possibility of loss of ascorbate during the preparation of microvessels should not be much as the isotonicity of the medium was maintained during the whole process. And, if there is any loss at all, it should be comparable in both the species as the same procedure of microvessel isolation was followed in both cases.

Oxidative stress has been considered to be a potential cause of endothelial aberration[22] especially in the brain microvessel endothelial cells which are very rich in polyunsaturated fatty acids compared to other parts of the brain.[23-24] The present study indicates that the presence of substantial amounts of both enzymatic and nonenzymatic antioxidants in the microvessels of monkey as well as rat may provide good protection to blood-brain barrier against oxidative stress. However, the primate has got a lower level of antioxidant protection showing its higher sensitivity to oxidative damage as compared to rats, a finding of the same type was observed in other organs also.[7,25,26] Primate were shown to be more susceptible for MPTP induced neurotoxicity also, due to less activity of MAO in their brain microvessels.[8]

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