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BLEOMYCIN-DETECTABLE IRON IN BRAIN TISSUE

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The normal brain contains regions with high concentrations of iron, part of which appears to be in a low molecular mass chelatable form. Iron complexes with a molecular mass of below 10,000, were measured in ultrafiltrates of homogenized gerbil brains using the bleomycin assay, and were found to average $20.5 \pm 3.5 \mu M$ ($n = 8$). As expected, no bleomycin detectable iron was found in the plasma of these animals.

No obvious difference in the tissue levels of bleomycin-detectable iron was recorded following ischaemia and reperfusion. This is probably due to the already abundant presence of iron in the brain and the likely release of iron from protected sites due to structural damage inherent in the preparative procedures used.

KEY WORDS: Bleomycin-iron, ischaemia-reperfusion, brain iron, low molecular mass iron.

INTRODUCTION

Low molecular mass iron complexes are essential for cell growth and viability. It has been proposed that such a pool of iron exists within cells for the synthesis of iron-containing proteins.[1] So far, the exact nature and amount of this iron pool remains unknown.[2] Complexable iron has the potential to stimulate oxygen radical reactions leading to damage at the molecular level, tissue degeneration and the amplification of disease processes [for reviews see references[3,4]]. Consequently, nature takes great care either to safely sequester iron in non- or poorly-reactive forms or to prevent the reaction of reduced oxygen intermediates with it.[5]

The brain contains high concentrations of iron, particularly in regions such as the globus pallidus, red nucleus and substantia nigra.[6-9] Part of this iron appears to be in a low molecular mass chelatable form since its removal or sequestration by iron chelators substantially inhibits the formation of oxygen radical reactions.[9-11] In this pilot study we attempt to ultrafilter and measure iron in gerbil brain that is available to complex to bleomycin.[12,13]

MATERIALS AND METHODS

Calf thymus DNA, ascorbate and bleomycin sulphate were from the Sigma Chemical Co., St Louis. All other chemicals were of the highest purity available from Aldrich Chemical Co., Milwaukee.

Preparation of Brain

The procedure developed by Chandler *et al.*[14] was followed in order to evaluate the role of ischaemia-reperfusion in the absence of anaesthetics. Briefly, male Mongolian gerbils, 95 ± 10 g, were obtained from Tumblebrook Farm, West Brook, MA. For surgery, gerbils were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). A ventral midline incision was made, and the common carotid arteries were exposed and isolated. (The Mongolian gerbil is unique in that cerebral ischaemia can be produced by occlusion of only the common carotid arteries). A loop of unwaxed dental floss (Johnson and Johnson) was placed around each artery. For each side, a 2 cm length of double lumen catheter (Dural Plastics and Engineering, Dural, N.S.W., Australia) was passed from the level of the carotid artery through the muscle layers and pushed through the shaved skin behind the gerbil's ear. The dental floss was then looped around the artery and each end inserted into a different hole of the catheter. The catheter was cemented to the skin and the dental floss was marked on the exterior to permit later estimation of each loop position. The incision was then closed.

After a two-day recovery period, occlusion of each carotid artery was produced by gently pulling the looped dental floss until the artery was completely occluded between the floss and the centre wall of the catheter. Heifitz clips were placed on the floss against the exterior end of the tubing to maintain occlusion. Release of the clips and partial insertion with tweezers of the dental floss resulted in reperfusion. This complete procedure was carried out in the unanaesthetized (awake) animal.

Those gerbils which received ischaemia only were decapitated while the carotid arteries were still occluded. After decapitation, the brains in all experiments were removed, washed in ice-cold 0.15 M NaCl and dissected free of blood vessels. The brain was weighed and homogenized on ice in specially cleaned glassware, washed in Chelex resin treated distilled water. The homogenizing buffer was Tris 0.17 M pH 7.4 from which contaminating iron had been removed by prolonged dialysis against conalbumin. The final brain-buffer dilution was 1 in 6. Blood samples were simultaneously collected from the animals into lithium heparin and spun at 3000 rpm to separate the plasma which was stored at −20°C until analysis.

Brain samples were centrifuged at 4°C for 30 min at a speed of 8000 rpm to clear the sample for ultrafiltration. The supernatant was loaded into a "Centricon 10" ultrafiltration cell and centrifuged at 4°C for 30 min at 14,000 rpm. The clear ultrafiltrate (approximately 0.5 ml) was used immediately for bleomycin-iron determinations.

Bleomycin Assay for Iron

The method has been described in detail elsewhere[13] but, briefly: 0.4 ml of DNA (1 mg/ml), 20 μl of bleomycin sulphate (1 unit/ml), 0.1 ml $MgCl_2$ (50 mM), 0.1 ml of sample or iron standard, 0.2 ml 1 M Tris buffer pH 7.4, and 0.1 ml ascorbate 7.5 mM were added to new clean plastic tubes and incubated at 37°C for 1 hour. Solutions of DNA, $MgCl_2$ and Tris buffer were treated by dialysis against conalbumin and, DNA and $MgCl_2$ were further treated with Chelex resin as previously described.[13] After incubation, 0.5 ml of thiobarbituric acid 1% w/v in 50 mM NaOH and 0.5 ml of HCl 25% v/v in distilled water were added to each tube and the tube contents heated at 100°C for 10 min. After colour development the tubes were cooled and the resulting pink chromogen was extracted into 1.5 ml of butan-1-ol. The tubes were centrifuged to separate the phases and the absorbance of the clear upper organic phase containing the chromogen was measured at 532 nm against appropriate standards and blanks.

RESULTS AND DISCUSSION

Chelatable iron complexes with a molecular mass below 10,000 Da have been measured in ultrafiltrates of homogenized gerbil brain using the bleomycin assay. Table I shows that no obvious change in complexable iron was detectable during the brief periods of ischaemia and reperfusion applied, although only small numbers of animals were studied. When all the results from the eight different animals are grouped together we obtain a mean value of 0.0205 μmole/g wet weight of brain (20.5 $\pm$ 3.5 μM) for bleomycin-detectable iron. As expected from previous work upon humans, no bleomycin-detectable iron was found in the plasma of gerbils. Indeed, the plasma retained considerable iron-binding capacity.

Ischaemia-reperfusion under similar conditions in gerbils has been previously shown to result in the generation of detectable oxygen radicals and to mediate behavioural changes in the gerbil.[15] It has been established that certain regions of the brain contain relatively high concentrations of non-heme iron with values greater that 3 mM quoted[6-9] and that complexable iron can be detected in cerebrospinal fluids in the low micromolar range.[16] It has been previously observed that some of the iron in brain homogenates and cerebrospinal fluids can bind to metal-binding agents.[9-12] This complexable low molecular mass iron in the brain is believed to play an important role in serotonin metabolism, since the binding of serotonin to serotonin-binding protein is thought to have an essential requirement for divalent iron ions.[17] Indeed, the unforeseen removal of complexable iron from the brain of patients with rheumatoid arthritis has resulted in loss of consciousness for periods up to 72 hours[18] implicating a central role for complexable iron in normal brain function.

Failure to detect any obvious changes in the low molecular mass iron content of gerbil brain tissue after brief periods of ischaemia-reperfusion (in only two experiments) may well reflect the already abundant presence of catalytic iron within the brain and the possible release of iron from protected sites as a result of structural damage inherent in the preparative procedures used here. Some of the reported deleterious events involving oxygen radicals in the brain following ischaemia and reperfusion probably result from the interaction of reduced oxygen intermediates with this readily available complexable iron.

TABLE I
Mean $\pm$SD of bleomycin-detectable iron 0.0205 μM/g wet weight $\pm$0.0035

Animal no	Procedure	Brain weight (g)	Bleomycin-detectable iron μmol/g wet weight	Bleomycin-detectable iron in plasma μM
1	No surgery	1.05	0.0193	Not detected
2	No surgery	1.12	0.0159	Not detected
3	5'ischaemia/ 6'reperfusion	1.06	0.0198	Not detected
4	5'ischaemia/ 6'reperfusion	1.02	0.0197	Not detected
5	5'ischaemia	1.09	0.0165	Not detected
6	5'ischaemia	1.04	0.0249	Not detected
7	No ischaemia No reperfusion	0.99	0.0228	Not detected
8	No ischaemia No reperfusion	0.96	0.0252	Not detected

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