Hemoglobin Potentiates Central Nervous System Damage

Sayed M. H. Sadrzadeh, Douglas K. Anderson, S. Scott Panter, Philip E. Hallaway, and John W. Eaton
Departments of Laboratory Medicine/Pathology and Medicine, and Dight Laboratories, University of Minnesota, Minneapolis, Minnesota 55455; and Departments of Neurology and Physiology, University of Cincinnati College of Medicine, and Neurology Service, Veterans Administration Medical Center, Cincinnati, Ohio 45220

Abstract

Iron and iron compounds—including mammalian hemoglobins—catalyze hydroxyl radical production and lipid peroxidation. To determine whether hemoglobin-mediated lipid peroxidation might be important in hemorrhagic injuries to the central nervous system (CNS), we studied the effects of purified hemoglobin on CNS homogenates and injected hemoglobin into the spinal cords of anesthetized cats. Hemoglobin markedly inhibits Na/K ATPase activity in CNS homogenates and spinal cords of living cats. Hemoglobin also catalyzes substantial peroxidation of CNS lipids. Importantly, the potent iron chelator, desferrioxamine, blocks these adverse effects of hemoglobin, both in vitro and in vivo. Because desferrioxamine is not known to interact with heme iron, these results indicate that free iron, derived from hemoglobin, is the proximate toxic species. Overall, our data suggest that hemoglobin, released from red cells after trauma, can promote tissue injury through iron-dependent mechanisms. Suppression of this damage by desferrioxamine suggests a rational therapeutic approach to management of trauma-induced CNS injury.

Introduction

The full consequences of trauma to the central nervous system (CNS) often are not evident until long after the primary insult. The sequellae of CNS injury may, in part, derive from exaggerated and prolonged inflammation. In experimentally induced trauma to feline spinal cord, marked accumulation of polymorphonuclear neutrophils (PMN)[1] only occurs in areas of hemorrhage (1), implying that interstitial blood somehow promotes CNS inflammation. Elements responsible for such amplified inflammation associated with hemorrhage are largely unknown.

We hypothesized that free hemoglobin (Hb) might be one magnifying factor in certain CNS inflammatory processes. Indeed, both simple iron salts and free Hb promote lipid peroxidation in vitro (2, 3). Such Hb-driven oxidation may also occur in vivo; hemorrhagic injury to the spinal cord leads to exaggerated release and peroxidation of CNS polyunsaturated fatty acids, generation of prostaglandins and leukotrienes (4), and extensive neuronophagia by PMN (1). In aggregate, the foregoing suggests that at least a fraction of the posttraumatic damage known to occur in CNS and, especially, spinal cord, may result from Hb-promoted peroxidation and inflammatory responses thereto. We have, therefore, carried out investigations to determine the possible deleterious effects of free Hb per se on CNS tissue in vitro and in vivo.

Methods

In vitro studies

Lipid peroxidation. Fresh feline spinal cord was collected by whole laminectomy (under anesthesia). Whole murine brain was excised < 30 s after cervical dislocation. CNS tissue was washed thoroughly in ice-cold saline and homogenized in 4 vol of 50 mM Tris buffer, pH 7.4, in an acid-washed Potter-Elvehjem homogenizer. Incubations were performed at 25°C in a total volume of 1 ml that contained 250 μl of diluted spinal cord or brain homogenate (protein concentration: 2.7 mg/ml), 50 nmol purified Hb (5) or metHb (as tetramer), 50 nmol $FeCl_2$, and 50 nmol desferrioxamine B. After incubation, the concentration of thiobarbituric acid–reactive substances (TBARS) in 10% trichloroacetic acid extracts was determined as previously described (6). Na/K ATPase activity was measured as described below.

Preparation of PMN. Human PMN were isolated by dextran sedimentation of whole heparinized blood followed by Ficol-Hypaque centrifugation as described earlier (7). Incubations were performed as described above and PMN were added to incubations (see above) in a final concentration of 10^7/ml.

In vivo model

Adult mongrel cats ranging in weight from 2 to 4 kg were used for these experiments. All cats were anesthetized with intraperitoneal injection of pentobarbital sodium (30 mg/kg) and intubated. Additional anesthesia, if necessary, was administered through a venous catheter. Cats were secured in a spinal stereotaxic frame and a one-segment laminectomy performed at L_4. The dura was incised and two 29-gauge needles stereotaxically inserted into both right and left anterior horns. The animals were injected with 500 nmol iron (5 μl of 100 mM $FeCl_2$ solution in saline), 110 nmol of Hb monomer (20 μl of 1.4 mM Hb tetramer), and the combination of Hb and desferrioxamine B (110 nmol and 18 μmol, respectively, in a final volume of 20 μl) over a period of 10 min. The incision was then closed. 24 h after injection, the incision was reopened and the L_4 spinal cord segment was frozen in situ with liquid nitrogen and removed with cold instruments under continuous liquid nitrogen irrigation. Uninjected sites distant from L_4 were frozen in situ and used as controls.

The gray matter of each frozen L_4 spinal cord segment was collected with a 2-mm stainless-steel dermatologic punch and weighed. Samples were then homogenized in 2 ml of 0.32 M sucrose in Hepes buffer, pH 7.4. Na/K ATPase activity was determined by the method previously described by Hunt and Craig (8) with minor modifications. Total protein was measured on each homogenate by the method of Lowry et al. (9).

Address reprint requests to Dr. Eaton, Department of Laboratory Medicine/Pathology, University of Minnesota, Box 198 UMHC, Minneapolis, MN 55455.

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1. *Abbreviations used in this paper:* Hb, hemoglobin; PMN, polymorphonuclear neutrophils; TBARS, thiobarbituric acid–reactive substances.

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Results

Both free iron and purified Hb, when added to homogenates prepared from feline spinal cord, cause brisk lipid peroxidation. As shown in Fig. 1 *A*, both iron and Hb promote formation of large amounts of TBARS (formerly called "malonaldehyde" and considered to be aldehydic by-products of the peroxidation of unsaturated fatty acids). Interestingly, the conversion of Hb to metHb does not influence the extent of resulting lipid peroxidation, suggesting that either valence will mediate the peroxidative reactions or that the metal can be converted to the active oxidation state by substances naturally occurring within CNS homogenates. This Hb-driven lipid peroxidation involves the participation of free iron because heme-free globin does not cause TBARS accumulation and the addition of desferrioxamine B blocks both the iron- and Hb-mediated reactions (Fig. 1 *A*).

In vivo, iron- and Hb-induced damage to the CNS might also involve the participation of inflammatory cells recruited to the site of hemorrhage. To test the possible synergistic effects of Hb and phagocytes, we incubated feline spinal cord homogenates with both Hb and added PMN. As shown in Fig. 1 *B*, Hb-driven lipid peroxidation is substantially increased by the addition of unstimulated human PMN. Note that this is a relatively small number of PMN, corresponding roughly to that present in a similar volume of whole blood (and far less than that usually found within inflammatory lesions in the CNS [10]).

Incubation of CNS homogenates with purified Hb will cause damage to other cellular components. Thus, as shown in Fig. 2, incubation of murine brain homogenates with either free iron or Hb leads to simultaneous lipid peroxidation (Fig. 2 *A*) and profound inhibition of neuronal Na/K ATPase activity (Fig. 2 *B*). As described below, similar effects of iron and Hb are also observed in vivo.

The above results support, but do not prove, the hypothesis that free Hb potentiates tissue damage arising from CNS hemorrhage. As a more direct test, we used feline spinal cord as a model of CNS trauma. In these experiments, spinal cords of anesthetized and laminectomized cats were stereotaxically injected with iron salts or purified Hb. Injection of $FeCl_2$, as expected (11), causes a substantial reduction in the activity of Na/K ATPase (Table I). In three separate animals, the injection of purified Hb caused a similar, but smaller, decrement in Na/K ATPase activity. The lesser magnitude of the Hb-mediated Na/

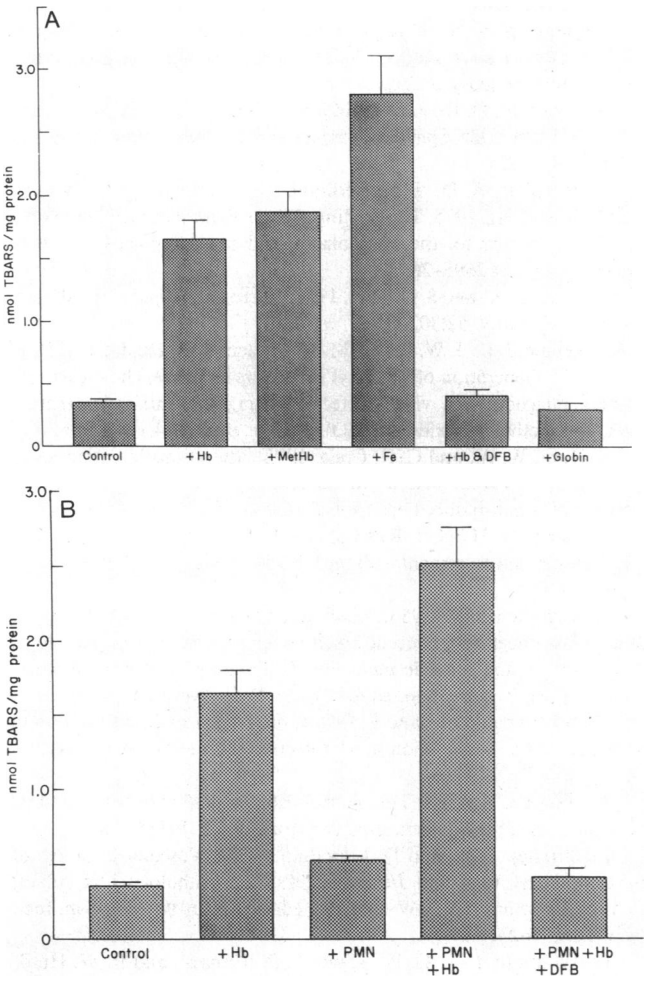

Figure 1. Hb-mediated lipid peroxidation in feline spinal cord homogenates. A. Homogenates of fresh spinal cord (protein concentration: 2.7 mg/ml), were incubated with purified Hb, metHb (as tetramer), $FeCl_2$, and desferrioxamine B (DFB). Following 60 min incubation, the concentration of TBARS was determined as previously described (2). B. Enhancement of Hb-mediated lipid peroxidation in feline spinal cord homogenates by added PMN.

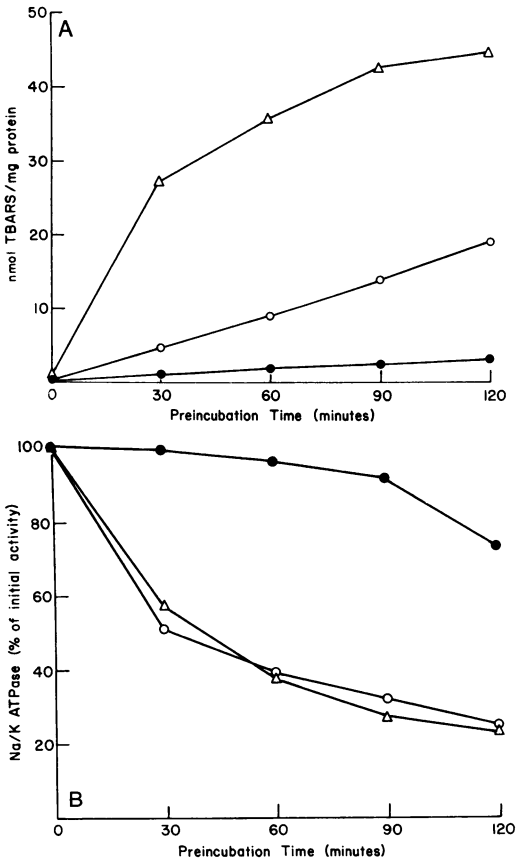

Figure 2. Hb- and iron-mediated lipid peroxidation (A) and inhibition of Na/K ATPase (B) in murine brain homogenates (protein concentration: 2.7 mg/ml). Analyses of TBARS and Na/K ATPase activity were performed on subsamples of simple incubation mixtures. (●) = homogenate with no addition; (○) = homogenate incubated with added purified Hb; and (△) = homogenate incubated with added $FeSO_4$. Each point represents the mean of values derived from three independent experiments.

K ATPase inhibition may be explained by our having injected roughly five times more free iron (500 nmol) than Hb iron (110 nmol). Unfortunately, simultaneous measurements of TBARS production in the injected areas were impossible due to the rapid clearance of this compound (11).

Two lines of evidence support the direct involvement of Hb iron in these pathologic effects. First, injection of similar amounts of globin freed of heme by acid acetone precipitation and washing causes no decrement in Na/K ATPase activity. Second, simultaneous injection of the potent iron chelator, desferrioxamine B, completely abrogates the Hb-induced inhibition of Na/K ATPase both in vitro (data not shown) and in vivo (Table I). This drug is known to chelate only free ferric iron and probably does not interact with heme iron (12).

Discussion

In cases of severe trauma to either brain or spinal cord, hemorrhage, with consequent destruction of extravasated red cells, is usually observed. Indeed, upon postmortem of previously traumatized humans, iron-rich lesions in the brain are often evident months later (13). The possible importance of iron compounds in the pathogenesis of CNS damage has not escaped attention. Indeed, epileptiform seizures and electroencephalographic abnormalities can be experimentally produced by the simple intracerebral instillation of either iron salts or Hb (14, 15). Furthermore, impaired clearance of Hb from the CNS may be a factor in the etiology of certain forms of familial idiopathic epilepsy (3). In the present case, we find that iron and Hb damage the spinal cord in vivo, as reflected by marked inhibition of neuronal Na/K ATPase activity. Furthermore, in vitro, both Hb and iron cause spontaneous peroxidation of CNS lipid and inhibition of Na/K ATPase activity. This peroxidation is further magnified by the inclusion of small numbers of PMN. The involvement of Hb-derived iron in these events in vivo is supported by the ineffectiveness of heme-free globin and the prevention of Hb-driven reactions by the potent iron chelator, desferrioxamine B.

These results have several important implications for the therapy of hemorrhagic CNS trauma. First, and most obviously, the presence of interstitial blood in CNS wounds is hazardous. Second, brisk and spontaneous peroxidation of CNS lipid by free Hb, even in the absence of phagocytes, implies that agents that interfere with the function of inflammatory cells (such as steroids) may be only partially effective in prevention of CNS damage after trauma and hemorrhage. Finally, the complete prevention of Hb-mediated damage to CNS tissues by desferrioxamine B should prompt early and thorough clinical tests of this drug in patients with recent CNS trauma.

Table I. Inhibition of Feline Spinal Cord Na/K ATPase Activity after the In Vivo Injection of Iron and Hb

Samples	Na/K ATPase*	n
Control (laminectomy, sham injection)	116±6.1	3
$FeCl_2$	29±0.9‡	10
Hb	73±10.8§	3
Hb & desferrioxamine	118, 112	2
Globin	109, 116	2

* Values are expressed as nanomoles pyrophosphate per minute per milligram protein.
‡ Differs from control, $P < 0.001$ (Student's t test, two tailed).
§ Differs from control, $P < 0.01$ (Student's t test, two tailed).

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References

1. Means, E. D., and D. K. Anderson. 1983. Neurophagia by leukocytes in experimental spinal cord injury. *J. Neuropath. Exp. Neurol.* 42:707–719.

2. Sadrzadeh, S. M. H., E. Graf, S. S. Panter, P. E. Hallaway, and J. W. Eaton. 1984. Hemoglobin: a biologic Fenton reagent. *J. Biol. Chem.* 259:14354–14356.

3. Panter, S. S., S. M. H. Sadrzadeh, P. E. Hallaway, J. L. Haines, V. E. Anderson, J. W. Eaton. 1985. Hypohaptoglobinemia: Association with familial epilepsy. *J. Exp. Med.* 161:748–754.

4. Anderson, D. K., J. D. Saunders, L. L. Dugan, E. D. Means, and L. A. Horrocks. 1985. Spinal cord injury and protection. *Ann. Emergency Med.* 14:816–821.

5. Barksdale, A. D., B. E. Hedlund, B. E. Hallaway, E. S. Benson, and A. Rosenberg. 1975. The binding of azide to human methemoglobin A_4. Error analysis for the interpolative and noninterpolative method. *Biochemistry.* 14:2695–2699.

6. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* 52:302–310.

7. Repine, J. E., J. W. Eaton, M. W. Anders, J. R. Hoidal, and R. B. Fox. 1979. Generation of hydroxyl radical by enzymes, chemicals, and human phagocytes in vitro: Detection using the anti-inflammatory agent—dimethyl sulfoxide (DMSO). *J. Clin. Invest.* 64:1642–1651.

8. Hunt, W. A., and C. R. Craig. 1973. Alterations in cation levels and Na^+, K^+-ATPase activity in rat cerebral cortex during the development of cobalt-induced epilepsy. *J. Neurochem.* 20:559–571.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

10. Oehmichen, M. 1983. Inflammatory cells in the central nervous system: an integrating concept based on recent research in pathology, immunology, and forensic medicine. *In* Progress in Neuropathology, Vol. 5. H. M. Zimmerman, editor. Raven Press, New York. 277–335.

11. Anderson, D. K., and E. D. Means. 1983. Lipid peroxidation in spinal cord: $FeCl_2$ induction and protection with antioxidants. *Neurochem. Pathol.* 1:249–264.

12. Keberle, H. 1964. The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann. NY Acad. Sci.* 119:758–768.

13. Brierley, J. B., and D. I. Grahum. 1984. Vascular disorders of the central nervous system. *In* Greenfield's Neuropathology. J. H. Adam, J. A. N. Corsellis, and L. W. Duchen, editors. John Wiley & Son, Inc., New York. 162–182.

14. Willmore, L. J., G. W. Sypert, J. B. Munson, and R. W. Hurd. 1978. Chronic focal epileptiform discharges induced by injection of iron into rat and cat cortex. *Science (Wash. DC).* 200:1501–1503.

15. Rosen, A. D., and N. V. Frumin. 1979. Focal epileptogenesis after intracortical hemoglobin injection. *Exp. Neurol.* 66:277–284.