Iron-induced oxidative brain injury after experimental intracerebral hemorrhage

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Summary

We investigated the occurrence of DNA damage in brain after intracerebral hemorrhage (ICH) and the role of iron in such injury. Male Sprague-Dawley rats received an infusion of 100 μL autologous whole blood or 30 μL FeCl2 into the right basal ganglia and were sacrificed 1, 3, or 7 days later. 8-hydroxyl-2′-deoxyguanosine (8-OHdG) was analyzed by immunohistochemistry, while the number of apurinic/apyrimidinic abasic sites (AP sites) was also quantified. 8-OHdG and AP sites are two hallmarks of DNA oxidation. DNA damage was also examined using PANT and TUNEL labeling. Dinitrophenyl (DNP) was measured by Western blot to compare the time course of protein oxidative damage to that of DNA. DNA repair APE/Ref-1 and Ku-proteins were also measured by Western blot. Bipyridine, a ferrous iron chelator, was used to examine the role of iron in ICH-induced oxidative brain injury.

An increase in 8-OHdG, AP sites, and DNP levels, and a decrease in APE/Ref-1 and Ku levels were observed. Abundant PANT-positive cells were also observed in the perihematomal area 3 days after ICH. Bipyridine attenuated ICH-induced changes in PANT and DNP. These results suggest that iron-induced oxidation causes DNA damage in brain after ICH and that iron is a therapeutic target for ICH.

Keywords: Intracerebral hemorrhage; iron; oxidative DNA injury; 8-OHdG; AP sites; DNP; PANT; APE/Ref-1; Ku-proteins; brain edema.

Introduction

Intracerebral hemorrhage (ICH) is a common and often fatal subtype of stroke. Iron is one of the hemoglobin degradation products and iron overload in the brain can cause free radical formation and oxidative damage such as lipid peroxidation after ICH [12]. There are several potential targets for oxidative damage following ICH.

We hypothesized that iron-induced oxidative DNA damage occurs after ICH and that it contributes to ICH-induced brain injury. Formation of the DNA modification 8-hydroxyl-2′-deoxyguanosine (8-OHdG) and apurinic/apyrimidinic (AP) sites are 2 oxidative DNA injury markers [5, 7]. This study examines the effect of ICH and intracerebral infusion of iron on these 2 parameters. Whether DNA damage might result in single and double strand breaks was examined using PANT and TUNEL staining, respectively [12]. The time course of DNA oxidative damage was compared to that in proteins using an anti-dinitrophenyl (DNP) antibody which can be used to detect protein oxidation [8]. AP endonuclease (APE)/Ref-1 and Ku-proteins are multifunctional proteins associated with DNA repair but which are decreased following cerebral ischemia [6, 7]. In addition, we examined the effect of bipyridine, a ferrous iron chelator, on ICH-induced oxidative brain injury.

Materials and methods

Animal preparation and experimental groups

Animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Male Sprague-Dawley rats, each weighing 300 to 400 g, were used for all experiments. The animals were anesthetized with pentobarbital (40 mg/kg i.p.) and the right femoral artery was catheterized to sample blood for intracerebral infusion. The rats were positioned in a stereotaxic frame and a 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma). Autologous whole blood (100 μL) or FeCl2 (10 mM, 30 μL) was infused at a rate of 10 μL/min with the use of a microinfusion pump.

This study was performed in 3 parts. Part 1 evaluated the time course of iron accumulation, oxidation, and DNA injury after ICH. Iron accumulation around hematoma was measured by Perl’s iron staining. 8-OHdG were investigated by immunohistochemistry (n = 3 each time point). The number of AP sites was measured quantitatively (n = 3–6 each time point). DNP, APE/Ref-1, and Ku-proteins were investigated by Western blot analysis (n = 3 each time point). TUNEL and PANT staining investigated the time course of DNA damage (n = 3 each time point).

Part 2 examined the effect of iron on oxidation and DNA damage. In this part, rats received an intracaudate injection of FeCl2 or a nee-
dle insertion (n = 3 each time point). The rats were sacrificed 24 hours later.

Part 3 investigated the effect of bipyridine (2,2’-dipyridyl) on DNP and PANT staining. Animals were immediately treated with either bipyridine (25 mg/kg in 1 mL saline i.p. per 12 hours) or vehicle (1 mL saline i.p. each time) after ICH.

Iron staining (Perl’s) and immunohistochemistry (8-OHdG)

For detection of ferric iron, a modified Perl’s staining was performed [9]. In immunostaining, the avidin-biotin complex technique was used. The primary antibody was mouse anti-8-OHdG monoclonal antibody (10 μg/mL) purchased from Oxis International Inc. (Portland, OR). The second antibody was anti-mouse IgG antibody (1:150) (Vector Laboratories, Burlingame, CA).

Detection of AP sites in DNA

DNA extraction was performed using a DNA isolation kit produced by Dojindo Molecular Technologies Inc. (Gaithersburg, MD). The aldehyde reactive probe (ARP) labeling and quantification of AP sites were performed by the AP sites assay kit (Dojindo). The ARP-labeled DNA was quantified using a 96-well microplate, similar to an enzyme-linked immunoabsorbent assay study. The wells were subjected to optical density measurement at 630 nm. ARP assays were performed in triplicate and the means were calculated. The data, expressed as the number of AP sites per 100,000 nucleotides, were calculated based on the linear calibration curve generated for each experiment using ARP-DNA standard solutions.

Western blot analysis (DNP, APE/Ref-1, and Ku-proteins)

Briefly, 50 μg proteins for each were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham, Piscataway, NJ). Membranes were probed with a 1:1000 dilution of the primary antibody and a 1:1500 dilution of the second antibody (BIO-RAD Laboratories, Hercules, CA). The antigen-antibody complexes were visualized with a chemiluminescence system (Amersham) and exposed to film. The relative densities of bands were analyzed with NIH Image (Version 1.61, National Institutes of Health, USA).

Detection of DNA single- and double-strand breaks by PANT and TUNEL staining

PANT and TUNEL staining were performed on adjacent brain sections to detect DNA single- and double-strand breaks according to the method described by Wu et al. [12].

Statistical analysis

All data in this study are presented as mean ± SD. Data were analyzed using analysis of variance, followed by Scheffe’s post hoc test. Significance levels were measured at p < 0.05.

Results

Time course of iron accumulation, oxidation DNA injury after ICH

Table 1 shows the summary of changes in some parameters associated with iron accumulation, oxidation DNA injury after ICH. An increase in 8-OHdG, AP sites, and DNP levels, and a decrease of APE/Ref-1 and Ku levels were observed in the ipsilateral basal ganglia, especially 3 days after ICH. Abundant PANT-positive cells were also observed in the perihematomal area 3 days after ICH.

Influence of iron on oxidation and DNA damage

DNP protein levels in the ipsilateral basal ganglia after ferrous iron injection were increased compared with the sham ipsilateral and the Fe²⁺ injection-contralateral basal ganglia 24 hours after Fe²⁺ injection (p < 0.01, Fig. 1A). PANT-positive cells were also detected in the ipsilateral basal ganglia 24 hours after Fe²⁺ injection. There were no PANT-positive cells in the contralateral basal ganglia or in the sham-ipsilateral basal ganglia (Fig. 1B).

Effect of bipyridine, a ferrous iron chelator, on ICH

Bipyridine treatment given immediately after ICH reduced DNP protein levels in the ipsilateral basal ganglia compared to vehicle-treated animals (3 days post-ICH, p < 0.01; Fig. 2A). Similarly, while PANT-positive cells were detected in the perihematomal area in vehicle-treated rats at 3 days after ICH (Fig. 2B-b), with bipyridine treatment given immediately after ICH, there were no PANT-positive cells detected in the ipsilateral basal ganglia (Fig. 2B-c).
Fig. 1. (A) Western blot analysis showing the DNP concentration in the sham-ipsilateral (lanes 1–3), the Fe$^{++}$ injection-ipsilateral (lanes 4–6), and the Fe$^{++}$ injection-contralateral (lanes 7–9) basal ganglia 24 hours. Equal amounts of protein (50 µg) were used. (B) PANT staining in the sham-ipsilateral (a), Fe$^{++}$ injection-ipsilateral (b), and Fe$^{++}$ injection-contralateral (c) basal ganglia 24 hours after ICH. Bar = 20 µm.

Fig. 2. The effect of bipyridine treatment (25 mg/kg i.p. given immediately after ICH) on DNP expression following ICH. (A) Western blot analysis showing DNP concentration in the vehicle-contralateral (lanes 1–3), vehicle ipsilateral (lanes 4–6), and bipyridine treatment-ipsilateral (lanes 7–9) basal ganglia 3 days after ICH. Equal amounts of protein (50 µg) were used. (B) PANT staining in the vehicle-contralateral (a), vehicle-ipsilateral (b), and bipyridine treatment-ipsilateral (c) 3 days after ICH. Bar = 20 µm.
**Discussion**

**Iron accumulation and oxidative DNA damage after ICH**

Although iron is essential for normal brain function, iron overload can cause brain injury [2]. After ICH, iron concentrations in the brain can reach very high levels. In the present study, iron-positive cells were found in the perihematomal area as early as the first day, detected by Perl’s staining. DNA is vulnerable to oxidative stress, and 8-OHdG and AP sites are sensitive markers of such DNA injury [5]. Normally, APE, a DNA repair enzyme, repairs AP sites in DNA [7]. Ku-proteins are also DNA repair proteins [6]. APE and Ku is constitutively expressed in the non-injured brain, but can decrease due to oxidative DNA damage [6, 7]. DNA damage can result from at least 2 pathways: endonuclease-mediated DNA fragmentation or oxidative injury [3]. In the present study, we could not find obvious double-strand breaks by the TUNEL method, but did detect single-strand breaks by PANT staining. We also found abundant positive cells around the hematoma 3 days after ICH. As with the 8-OHdG immunoreactivity and AP sites results, this may reflect oxidative damage. The oxidative proteins are analyzed for carbonyl content by immunoblot with anti-DNP antibody and a specific band could be detected at 50 kDa [10]. The DNP protein levels and the number of AP sites and 8-OHdG immunoreactivity all peaked at 3 days after ICH in this study. These results suggest that brain oxidative damage peaks about 3 days after ICH.

**Influence of iron on brain oxidation and DNA damage**

It is known that iron can react with lipid hydroperoxides to produce free radicals, which contribute to neuronal damage during ischemia/reperfusion [11]. In vitro, exposure to FeSO₄ results in lipid peroxidation in neurons and an increase in apoptotic cell death [13]. The present study shows that infusion of FeCl₂ into the caudate induces DNP expression and PANT-positive cells 24 hours later, suggesting the Fe²⁺ might contribute to ICH-induced oxidative stress and DNA damage, a hypothesis supported by our findings on the effects of bipyridine.

**Effect of iron chelation on ICH**

Bipyridine is a small molecular weight (MW 220) ferrous iron chelator. It is hydrophobic, so that at physiological pH it partitions into cell membranes and binds iron as it passes through this lipid environment [1]. In a previous study, we found that deferoxamine, a ferric iron chelator, attenuates brain edema and neurological deficits in a rat ICH model [9]. These results suggest that both ferrous and ferric iron chelators such as bipyridine and deferoxamine could be useful for the treatment of brain edema following ICH. Because of their permeability, both bipyridine and deferoxamine are capable of chelating intracellular iron, although deferoxamine also chelates extracellular iron [4].

**Conclusion**

The present study suggests that iron-induced oxidation causes DNA damage in the brain following ICH. Oxidative stress and iron chelation are potential therapeutic targets for ICH.

**References**


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