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Iron enhances the neurotoxicity of amyloid β

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Abstract

Brain microbleeds often occur in Alzheimer's disease patients. Our previous studies have demonstrated that iron contributes to brain injury following intracerebral hemorrhage. This study investigated the effect of iron on amyloid β ($A\beta$)-mediated brain injury. There were two parts to this study. In first part, rats received an intracaudate injection of saline, iron, $A\beta$ 25–35 or iron + $A\beta$ 25–35. In the second part, rats received intracaudate injection of iron + $A\beta$ and were treated with saline or cystamine, an inhibitor of transglutaminase. Rats were killed after 24 hours for brain edema measurement. DNA damage, neuronal death and tissue-type transglutaminase (tTG) expression were also examined. We found that brain water content in the ipsilateral caudate was higher ($p < 0.05$) in rats injected with iron + $A\beta$ than with iron, $A\beta$ or saline. Combined iron + $A\beta$ injection also resulted in more severe DNA damage (both single- and double-strand; $p < 0.01$) and more Fluoro-Jade C staining ($p < 0.05$). Expression of tTG increased markedly in the iron + $A\beta$ group ($p < 0.05$) and treatment with a tTG inhibitor reduced brain edema ($p < 0.05$) and reduced degenerating neurons (124 ± 25 vs. $249 \pm 50/\text{mm}^2$ in vehicle-treated group, $p < 0.05$). These results suggest that increased brain iron from microbleeds may exaggerate brain $A\beta$ toxicity and that tTG is involved in the enhanced toxicity.

Keywords

cerebral hemorrhage; iron; amyloid β ; brain edema; tissue-type transglutaminase

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and the most common cause of dementia. Although the precise mechanisms which cause AD are still unclear, amyloid β ($A\beta$) deposition may be involved in AD pathogenesis (1).

Studies have shown that the prevalence of brain microbleeds in AD patients is very high (2, 3). The effect of microbleeds on brain injury in AD patients needs to be examined, but intracerebral hemorrhage (ICH) can result in brain iron overload and brain iron accumulation has an important role in ICH-induced brain damage (4). We have demonstrated that deferoxamine, an iron chelator, reduces brain injury after ICH (5, 6). Evidence also shows that iron overload and oxidative stress occur in AD and deferoxamine can reduce the rate of decline of daily living skills in AD patients (7, 8).

A potential link between AD- and ICH-induced injury is the transglutaminases. These are a family of cross-linking enzymes catalyzing the formation of γ -glutamyl- ϵ -lysine bonds. Tissue-type transglutaminase (tTG) is abundantly expressed in brain and has a role in extracellular matrix development (9). An increase in tTG levels has been observed during

normal aging in humans (10). It has a role in brain injury after ICH and AD (8, 11). In the present study we examined the effect of iron on the neurotoxicity induced by A β . The role of tTG in A β - and iron-induced brain injury was also examined.

Materials and Methods

Animal Preparation and Intracerebral Injection

The University of Michigan Committee on the Use and Care of Animals approved the protocols for these animal studies. A total of 76 adult male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) weighing 275 to 300 g were used in this experiment. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the right femoral artery was catheterized to monitor arterial blood pressure, blood pH, PaO₂, PaCO₂, hematocrit and glucose levels. Core body temperature was maintained at 37.5°C with a feedback-controlled heating pad. Rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA), a cranial burr hole (1 mm) drilled and a 26-gauge needle inserted stereotaxically into the right caudate nucleus (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma). Saline, FeCl₂, A β or A β +FeCl₂ was injected at a rate of 2 μ l/min into the right caudate using a microinfusion pump. The needle was then removed and the skin incision sutured closed.

Experimental Groups

This study included two parts. The first part investigated whether iron enhances A β -induced neurotoxicity. The second part examined the role of transglutaminase in the brain injury induced by iron and A β .

In the first part, rats received an injection of 20 μ l saline, FeCl₂ (4 nmol), A β (25–35; 20 nmol, Bachem, Torrance, CA, USA) or FeCl₂ (4 nmol) + A β (20 nmol) into the right basal ganglia. All rats were killed 24 h after injection. The brains were sampled for edema measurement (n=6, each group) or histological examination (n=5, each group).

In the second part, rats received an injection of 20 μ l saline containing FeCl₂ (4 nmol) with A β (20 nmol). Two hours after the intracerebral injection, they were treated with an inhibitor of tissue-type transglutaminase (tTG), cystamine dihydrochloride (Sigma-Aldrich Inc., St. Louis, MO, USA; 100 mg/kg, i.p.) or vehicle. All rats were killed at 24 h after intracerebral injection. Brains were sampled for edema measurement (n=11, each group) or histological examination (n=5, each group).

Brain Water Content Measurement

Animals were anesthetized and decapitated to measure brain water content (12). Brains were removed and a coronal tissue slice (3-mm thickness) 4 mm from the frontal pole was cut using a blade. The brain tissue slice was divided into two hemispheres along the midline, and each hemisphere was dissected into cortex and basal ganglia. The cerebellum served as a control. Three tissue samples from each brain were obtained: the ipsilateral and contralateral basal ganglia, and the cerebellum. Brain samples were immediately weighed on an electric analytical balance (model AE 100; Mettler Instrument, Highstown, NJ, U.S.A.) to obtain the wet weight. Brain samples were dried at 100°C for 24 hours to obtain the dry weight. The water content was determined as (Wet Weight - Dry Weight)/Wet Weight.

Immunohistochemistry for tissue transglutaminase (tTG)

Immunohistochemistry was performed to detect tTG expression (11). Briefly, rats were anesthetized and underwent intracardiac perfusion with 4% paraformaldehyde in 0.1 M PBS. Brains were removed and kept in 4% paraformaldehyde for 12 h, then immersed in 25%

sucrose for 3–4 days at 4°C. Brains were then embedded and sectioned on a cryostat (18 mm thick). Immunohistochemistry staining was performed using the avidin-biotin complex technique. The primary antibody was monoclonal mouse anti-tTG (NeoMarkers, Fremont, CA, USA; 1:1000 dilution). The secondary antibody was horse anti-mouse (Abgent Inc., San Diego, CA, USA; 1:600 dilution). Normal mouse IgG served as a negative control.

Fluoro-Jade C staining

To assess neuronal degeneration, Fluoro-Jade C staining was performed on brain coronal sections (11).

Polymerase I-mediated biotin-dATP nicktranslation (PANT) staining

PANT staining was performed on adjacent brain sections to detect DNA single-strand breaks using a previously described method (13). Slides were fixed in 4% paraformaldehyde for 15 minutes and washed with PBS. 1% Triton-X-100 was used to permeabilize sections and 2% H₂O₂ to quench endogenous peroxidases. After washing with PBS, slides were incubated in a moist chamber at 37°C for 90 min with PANT reaction mixture (5 mM MgCl₂, 10 mM 2-mercaptoethanol, 20 µg/ml bovine serum albumin, dGTP, dCTP, and dTTP at 30µM each, 29 µM biotinylated dATP, 1 µM dATP, and 40 U/ml Escherichia coli DNA polymerase I (GIBCO BRL) in PBS (pH 7.4). The slides were incubated in streptavidin-horseradish peroxidase (Vectastain Elite ABC) in PBS containing bovine serum albumin for 90 min at room temperature. Detection of the biotin-streptavidin-peroxidase complex was carried out by incubating slides with DAB in 0.1M PBS and 0.05% H₂O₂. Slides incubated with reaction mixture without DNA polymerase were used as controls.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL staining was performed (13) using a ApopTag Peroxidase Kit (Intergen). First, 3 % hydrogen peroxide in 0.1MPBS was applied to slides for five minutes to quench endogenous peroxidases. After washing with PBS and equilibrating with the solution supplied, the specimens were incubated with TdT enzyme at 37°C for 1 hour. The reaction was stopped by washing with buffer for 10 minutes. Anti-digoxigenin peroxidase conjugate was then applied for 30 minutes at room temperature. The omission of the terminal deoxynucleotidyl transferase served as the negative control.

Cell counts

For cell counting, we used 18 µm thick coronal sections from either 1 mm anterior or 1 mm posterior to the injection site. Three high-power images (× 40 magnification) were taken in the ipsilateral basal ganglia using a digital camera. Fluoro-Jade C, PANT, TUNEL and tTG positive cells were counted on these 3 areas from each rat brain section (Figure 1) by a blinded observer.

Statistical analysis

All data in this study are presented as mean±SD. Data were analyzed with Student's t-test or one-way analysis of variance (ANOVA). Differences were considered significant at p<0.05.

Results

Physiological parameters were recorded immediately before intracerebral injections. The mean arterial blood pressure (MABP), blood pH, blood gases, hematocrit and blood glucose were in normal ranges (MABP, 80–120 mm Hg; pO₂, 80–120 mm Hg; pCO₂, 35–45 mm Hg; hematocrit, 38–42%; blood glucose, 80–120 mg/dl).

To examine whether or not iron can enhance brain edema induced by A β , rats were given injections of saline, iron, A β or iron+A β into the right basal ganglia. While iron and A β did not cause marked brain edema in the ipsilateral basal ganglia at 24 hours (78.6 ± 0.6 and $78.4\pm0.3\%$ vs. $78.4\pm0.5\%$ in saline group, $p > 0.05$, Figure 2), co-injection of iron and A β showed a significant increase of brain edema ($79.7\pm1.0\%$, $p<0.05$ vs. other groups, Figure 2).

Fluoro-Jade C was chosen to detect degenerating neurons (Figure 3). Intracaudate injection of saline caused minimal neuronal death. While, injection of iron or A β alone caused some neuronal death in the ipsilateral basal ganglia, co-injection of iron+A β resulted in more neuronal death than all other groups ($222 \pm 31/\text{mm}^2$, $p<0.05$; Figure 3).

Neuronal death can result from DNA damage. Whether or not iron+A β causes DNA damage was examined using PANT staining and TUNEL staining. Intracerebral injection of iron did not cause significant single-strand DNA damage (PANT positive, Figure 4). A β injection alone increased the number of PANT positive cells in the ipsilateral basal ganglia (237 ± 57 vs. $96\pm70/\text{mm}^2$, $p<0.05$), but iron potentiated the neuronal toxicity of A β . The number of PANT positive cells in the iron+A β group was $548\pm97/\text{mm}^2$, Figure 4). The number of TUNEL positive cells in the ipsilateral basal ganglia in iron+A β group was also much higher than in the other groups (e.g., 584 ± 169 vs. $184\pm92/\text{mm}^2$ in A β alone group, $p<0.05$, Figure 5).

Expression of tTG in the brain is associated with neurodegeneration. There were tTG positive cells in the ipsilateral basal ganglia of rats that received saline, iron and A β , but injecting iron+A β caused a significant increase of tTG positive cells (e.g. 296 ± 42 vs. $119\pm56/\text{mm}^2$ in the A β group, $p<0.05$, Figure 6) at 24 hours.

To clarify whether tTG plays a role in the brain injury caused by iron+A β , the effects of cystamine, an inhibitor of tTG, on brain edema formation and neuronal death were examined. Rats treated with cystamine 2 hours after intracerebral injection of iron+A β had less brain edema (78.3 ± 0.5 vs. $79.2\pm1.1\%$ in vehicle-treated group, $p<0.05$, Figure 7A) and fewer Fluoro-Jade C positive cells (124 ± 25 vs. $249\pm50/\text{mm}^2$, $p<0.05$, Figure 7B) in the ipsilateral basal ganglia.

In all experiments, no marked brain injury was found in the contralateral basal ganglia.

Discussion

There were several findings in this study: 1) Intracerebral injection of iron and A β caused marked brain edema; 2) Iron potentiated A β -induced DNA damage and neuronal death; 3) Co-injection of iron and A β resulted in an increase of tTG expression; and 4) Cystamine, an inhibitor of tTG, reduced the brain edema and neuronal death induced by iron+A β . These results suggest that iron can enhance A β neurotoxicity, an effect partially mediated by tTG.

Iron overload plays an important role in ICH-induced brain injury (4). After erythrocyte lysis, iron concentrations in the brain can reach very high levels. Our previous study showed an increase of brain non-heme iron after intracerebral hemorrhage in rats (14). Iron deposition in the brain has been found in several animal ICH models (5, 15) and results in oxidative brain injury (16). Intracerebral infusion of iron causes brain damage and deferoxamine, an iron chelator, reduces hematoma- and hemoglobin-induced brain injury (4).

Iron accumulation also occurs in the AD brain and has a role in the progression of AD (7). There are many factors that may cause iron accumulation in AD brain but microbleeds

may have a special role. The morbidity of brain microbleeds in AD patients is very high. T2* magnetic resonance imaging is very sensitive method to detect iron deposition and recent T2* studies showed that microbleeds occur in 29–32% of AD patients (2, 3). It is important to understand whether or not microbleeds may exaggerate brain injury in AD patients. Both iron and A β can cause oxidative stress and our present study showed that iron can potentiate neurotoxicity of A β . It is known that ferrous and ferric iron react with lipid hydroperoxides to produce free radicals (17) and antioxidants block neuronal toxicity induced by hemoglobin and iron (18, 19).

A pathological hallmark of AD is the presence of characteristic deposits in the brain, known as amyloid plaques. A β peptide is the key element of amyloid plaque (20). Accumulation of extracellular A β peptide in the brain has been proposed to trigger major pathologies in AD patients. Aggregation of monomeric A β forms neurotoxic A β aggregates, such as oligomers, protofibrils and mature fibrils. It has been reported that the aggregation of the A β peptide may be driven by interaction between A β and metal ions (21) and iron can potentiate A β toxicity *in vitro* (22). Our present results demonstrated that iron can potentiate A β toxicity *in vivo*. Combined injection of iron and A β (25–35) not only induced brain edema but also exaggerated single- and double-strand DNA damage and neurodegeneration.

It is unclear why iron potentiates neuronal toxicity of A β . Our results showed that combined injection of iron and A β but not iron or A β alone increases tTG expression in the ipsilateral basal ganglia. Tissue-type transglutaminase is one of nine transglutaminases and is expressed in the central nervous systems and is localized mostly in the cytoplasmic compartment of neurons (9). Previous studies have indicated that tTG is involved in the development and pathology of AD (23) and also contributes to brain injury after ICH (11). Tissue-type transglutaminase levels and activity were elevated in AD brains and tTG immunoreactivity was found in both senile plaques and neurofibrillary tangles (24–26). tTG-catalyzed intermolecular cross-links induce stable, rigid and insoluble protein, similar to the protein aggregates observed at the site of pathology in neurodegenerative diseases. A β peptides are good substrates for tTG and tTG also catalyzes the formation of A β oligomers (27). Therefore, future studies should examine the role of tTG in the A β aggregation process.

To clarify the role of tTG in neurotoxicity induced by iron and A β , cystamine, an inhibitor of transglutaminase, was used. We showed that cystamine reduces both brain edema and neuronal death caused by combined injection of iron and A β . This result supports the hypothesis that tTG plays a role in the process whereby iron enhances the neurotoxicity of A β .

To our knowledge, this is the first proof of concept study examining the role of iron in A β -induced brain injury. It focused on only one peptide fragment of A β , A β 25–35, a fragment known to cause neurotoxicity and oxidative stress (28). Further studies are needed to examine the interactions between iron and other types of A β , including full length A β 1–42 (and scrambled peptides). In addition, this study focused on acute neurotoxicity. Further long-term studies are needed to test the role of iron in chronic neurodegeneration induced by A β and to elucidate the mechanisms of cystamine-induced neuroprotection.

In conclusion, iron increased brain tTG levels and enhanced the A β -induced acute neurotoxicity *in vivo*. Future studies should determine whether iron aggravates A β -induced chronic neurodegeneration.

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Figure 1.
The diagram showing 3 areas in the ipsilateral basal ganglia for cell counting.

Figure 2.

Brain water content 24 hours after injection of 20 μ l of saline, iron, A β or iron+A β into the right basal ganglia. Values are mean $\pm$ SD, n=6, # p<0.01 vs. the other groups.

Figure 3.

(A) Fluoro-Jade C positive cells in the basal ganglia 24 hours after injection of 20 μ l of saline, iron, A β or iron+A β . (B) Cell counting in the ipsilateral basal ganglia. Values are mean $\pm$ SD, n=5, # p<0.01 vs. the other groups, * p<0.05, ** p<0.01 vs. saline.

Figure 4.

(A) PANT positive cells in the basal ganglia 24 hours after an injection of 20 μ l of saline, iron, A β or iron+A β into the right basal ganglia. (B) Cell counting in the ipsilateral basal ganglia. Values are mean $\pm$ SD, n=5, # p<0.01 vs. the other groups, * p<0.05 vs. saline.

Figure 5.

(A) TUNEL positive cells in the basal ganglia 24 hours after an injection of 20μl saline, iron, Aβ or iron + Aβ into the right basal ganglia. (B) Cell counting in the ipsilateral basal ganglia. Values are mean ± SD, n=5, #p<0.01 vs. the other groups.

Figure 6.

(A) Immunohistochemistry showing tTG expression in the basal ganglia. Rats received an intracaudate injection of 20 μ l saline, iron, A β or iron + A β . (B) Cell counting in the ipsilateral basal ganglia. Values are mean $\pm$ SD, n=5, #p<0.01 vs. the other groups.

Figure 7. Brain water content (A) and Fluoro-Jade C positive cells (B) in the ipsilateral basal ganglia 24 hours after an injection of iron+A β (20 μ l) in rats treated with cystamine dihydrochloride (100mg/kg, i.p.) or vehicle. Values are mean $\pm$ SD, * p<0.05, # p<0.01 vs. vehicle-treated group.