Neurochemical Research, Vol. 10, No. 12, 1985, pp. 1635–1644

IRON UPTAKE BY GLIAL CELLS

KENNETH F. SWAIMAN AND VALYNDA L. MACHEN
Division of Pediatric Neurology
Department of Neurology
University of Minnesota
Minneapolis, Minnesota

Accepted June 3, 1985

Dynamic studies of iron metabolism in brain are generally unavailable despite the fact that a number of neurologic conditions are associated with excessive accumulation of iron in central nervous tissue. Cortical non-neuronal (glial) cultures were prepared from fetal mouse brain. After 13 days the cultures were exposed to radiolabeled iron. Brisk and linear total iron uptake and ferritin iron uptake occurred over 4 hours. When methylamine or ammonium chloride was added, (both known inhibitors of transferrin iron release because of their lysosomotropic properties), total iron uptake was diminished. Further studies indicated that methylamine inhibits glial cell ferritin iron incorporation. Glial cell iron transport is similar to previously reported neuronal cell iron transport (1) but glial cell iron uptake proceeds at a faster rate and is more susceptible to the inhibition of certain lysosomotropic agents. The data reinforces the likelihood that iron uptake by nervous tissues is transferrin-mediated.

INTRODUCTION

Brain iron metabolism is altered greatly in Hallervorden-Spatz syndrome (2–8). Abnormally increased iron accumulation also occurs in Parkinson disease (9–12), Alzheimer disease (13), Pick disease (14–15), general paresis (16), and cerebrohepatorenal disease (17). Deposits of iron in neurologic conditions accompanied by excess iron storage are usually largest in areas which normally manifest high iron concentration.

There has been virtually no information available concerning the rates of iron uptake by brain cells and the effect on brain cells of various known

Address reprint requests to: Dr. Kenneth F. Swaiman, Box 380 Mayo Building, 420 S.E. Delaware Street, Minneapolis, Minnesota 55455.

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blockers of iron transfer. Recent studies in our laboratory which demonstrated significant iron uptake by neuronal cultures (1) were followed by the experiments reported here. Mammalian non-neuronal (glial) cortical cultures were employed to delineate some details of iron transport into glial cells.

EXPERIMENTAL PROCEDURE

Glial Cell Cultures. The method of Olson and Holtzman (18) was modified and combined with that of Swaiman et al. (19). Newborn mice, 1–3 days old (22–24 days post-conception) were killed by asphyxiation and cervical dislocation. The cortices were dissected, minced, and dissociated by trypsinization and trituration.

Single cells were suspended in Eagle's minimal essential medium (MEM, GIBCO) modified to include 10% fetal calf serum (HyClone), 6 g glucose and 3.7 g $NaHCO_3$/L as well as penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension was diluted to 8×10^5 cells/ml. Subsequently, 0.3 ml of this inoculum was pipetted into 16 mm wells in a 24 well cluster tray. Material from 3 cortices was usually sufficient to plate 16 trays.

The cultures were incubated at 37°C in a water-saturated 10% carbon dioxide atmosphere. The medium was changed at 24 hours, 48 hours, and every 3–4 days thereafter. Beginning at 24 hours, 10% horse serum (HyClone); inactivated at 56°C for 30 min was substituted for the fetal bovine serum. The volume of medium added to each well was increased at 24 hours to 0.5 ml to discourage neuronal growth.

These cultures have been demonstrated to be virtually free of neuronal and fibroblast elements by the use of appropriate and specific glial staining (18) and by the absence of clonazepam-displaceable [^{3}H]diazepam binding after day 9 in culture (20). No morphologically identifiable neurons were present after day 9.

Morphologic development of the cultures was followed by photographing either living or glutaraldehyde-fixed cultures.

Radioisotope Preparation. ^{55}Fe solution (20 μg/ml; 20 μCi/μg) was obtained by dilution of $^{55}FeCl_3$ (27.03 mCi/mg; New England Nuclear) with a 10 μg/ml iron solution prepared from acid dissolved wire. Iron concentration was determined by the procedure of Nelson (21). An appropriate volume of horse serum (HyClone, lot #300381) was mixed with the radiolabeled iron stock solution, heated for 30 min at 37°C, and diluted with MEM to form a 10% horse serum/MEM preparation. Use of the same serum lot for all experiments assured comparable transferrin concentration and characteristics. Horse serum transferrin content was 331 mg/dl and the total iron binding capacity 462 μg/dl. Iron saturation was 48.9%.

Total Iron Uptake and Iron Blocker Studies. Methods adapted from the myocardial cell culture technique of Cox et al. (22) and reticulocyte study technique of Morgan (23) were used. Glial cell cultures were exposed 13 days after plating (34 days post-conception) to methylamine (CH_3NH_2) or NH_4Cl at concentrations of 5–15 mM.

Medium was routinely replaced in all wells 18–24 hours prior to the uptake study. CH_3NH_2 and NH_4Cl stock solutions (4.0 M) were prepared and adjusted to pH 7.3. At the initiation of the uptake study the medium in the culture wells was replaced with 250 μl 10% horse serum/MEM in control wells or 250 μl 10% horse serum/MEM supplemented with CH_3NH_2 or NH_4Cl in the desired concentrations in the experimental wells. The trays were placed in the incubator for 15 min at 37°C. ^{55}Fe in 10% horse serum/MEM (50 μl), prepared as described above, was added to the control wells, establishing a final iron concentration of 0.23 μg/ml (0.13μg/ml contributed by iron originally bound to the horse serum transferrin and

0.10 μg/ml subsequently added). A similar radiolabeled iron solution was supplemented with appropriate amounts of CH_3NH_2 or NH_4Cl and added to the experimental wells. The trays were replaced in the incubator for 1, 2, 3, and 4 hours. The cultures were washed 3 times with Ca^{+2}- and Mg^{+2}-free Hank's balanced salt solution (HBSS, GIBCO) adjusted to 330 mOsm with NaCl as necessary. The cells were removed with a total of 1.5 ml 0.2 N NaOH, protein determined by the method of Lowry et al. (24), and scintillation counting was performed after addition of 100 μl 3 N HCl and 10 ml Aquasol-2 (New England Nuclear).

The control condition and each concentration of CH_3NH_2 or NH_4Cl were represented by at least 4 wells at each time point. The data were expressed as the mean iron uptake (pmol Fe/mg protein) ± SEM; two separate dissections and experiments were performed which yielded essentially the same results. Significance tests were performed using the Student's *t* test.

Ferritin Iron Incorporation Studies. When cell culture material was used for ferritin iron incorporation studies the final HBSS wash was gently suctioned and the cells were frozen at −40°C. Radiolabeled iron incorporation into ferritin was assayed by a modification (1) of the method of Drysdale and Munro (25). After thawing, the cells were scraped from the wells, sonicated and 0.1 ml of water containing 0.1 mg of carrier horse ferritin was added. A 0.1 ml aliquot was taken for determination of total radiolabeled iron uptake; the remaining material was heated to 80°C for 20 min and then chilled and centrifuged at 3000 *g* at 4°C for 20 min. The supernatant was mixed with an equal volume of cold saturated ammonium sulfate and chilled overnight at 5°C. The ferritin precipitate was collected by centrifugation at 4200 *g* for 20 min at 4°C. The precipitate was dissolved in water (0.5 ml) and the uptake of radiolabeled iron into ferritin was determined by liquid scintillation counting.

The control condition and each concentration of CH_3NH_2 was represented by at least 4 wells at each time point. The data were expressed as the mean iron uptake (pmol Fe/mg protein) ± SEM; two separate dissections and experiments were performed which yielded similar results. Significance tests were performed using the Student's *t* test.

RESULTS

The inoculum consisted of both phase-bright and phase-dark cells about 6 μm in diameter. Within a day, the majority of cells had attached; some cells had flattened. The number of phase-bright cells was greatly reduced and cells which were stellate, boomerang-shaped, or spindle-shaped were dominant. By day 5 the general morphology of the cultures was characteristically glial; by day 9 no phase-bright cells or neuritic processes were seen. The other cells had grown in volume and were superimposed on a layer of confluent, flat, background cells. Although fine tapering processes extended as direct continuations of the apices of some spindle cells, these process were unbranched and appeared to taper and terminate within half a cell length. Between 9 and 18 days (Figure 1), general cellular patterns did not change, although further increases in cell size were seen.

Radiolabeled iron uptake was rapid and linear over the entire 4 hours (Figures 2 and 3); approximately 6 pmol/mg protein/hour. The plot was drawn by use of linear regression ($r = 0.99$). At 4 hours the SEM of total iron uptake was in the range of 4–8%.

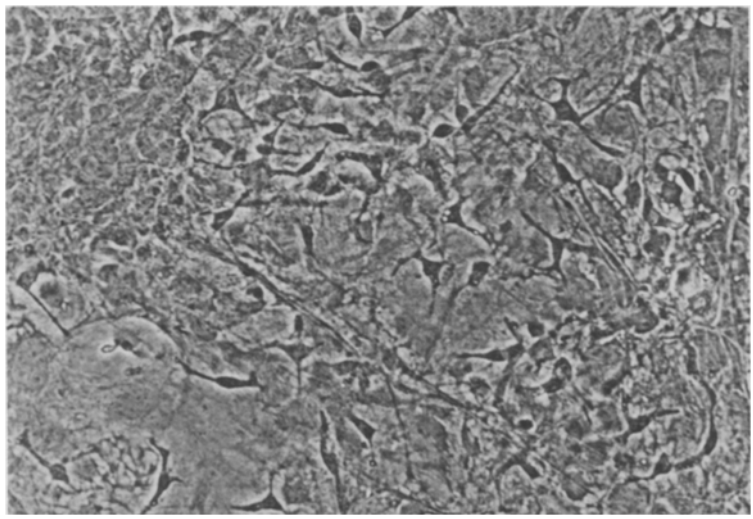

FIG. 1. Photomicrograph of 13 day non-neuronal (glial) cell culture depicting stellate and spindle-shaped glial cells. Short tapered processes extend from the apices of the stellate-shaped cells. (×200).

Inhibition by both CH_3NH_2 and NH_4Cl (5–15 mM) of total iron uptake over the 4 hour incubation period was evident. The differences between the experimental and control values at each time point were statistically significant ($P < 0.01$) for all concentrations of CH_3NH_2 and NH_4Cl. The degree of inhibition was clearly concentration-related by 2 hours ($P < 0.05$). Both substances were associated with similar degrees of inhibition. The degree of inhibition of total iron uptake was linear over the 4 hour incubation period (Figures 2 and 3).

Ferritin incorporation of iron over the same time period was also linear ($r = 0.98$; Figure 4). At 4 hours the SEM of iron uptake into ferritin was in the range of 5–12%. The ferritin-bound iron fraction ranged from 20–40% of the total iron. In the presence of CH_3NH_2, the differences between the controls and experimental values for each concentration at each time point were statistically significant ($P < 0.01$). By 3 hours, the degree of inhibition was clearly concentration-related ($P < 0.05$; Figure 4).

It is noteworthy that in the presence of CH_3NH_2, ferritin-bound iron accumulation over the 4 hour period was decreased commensurately with decrease in total iron uptake.

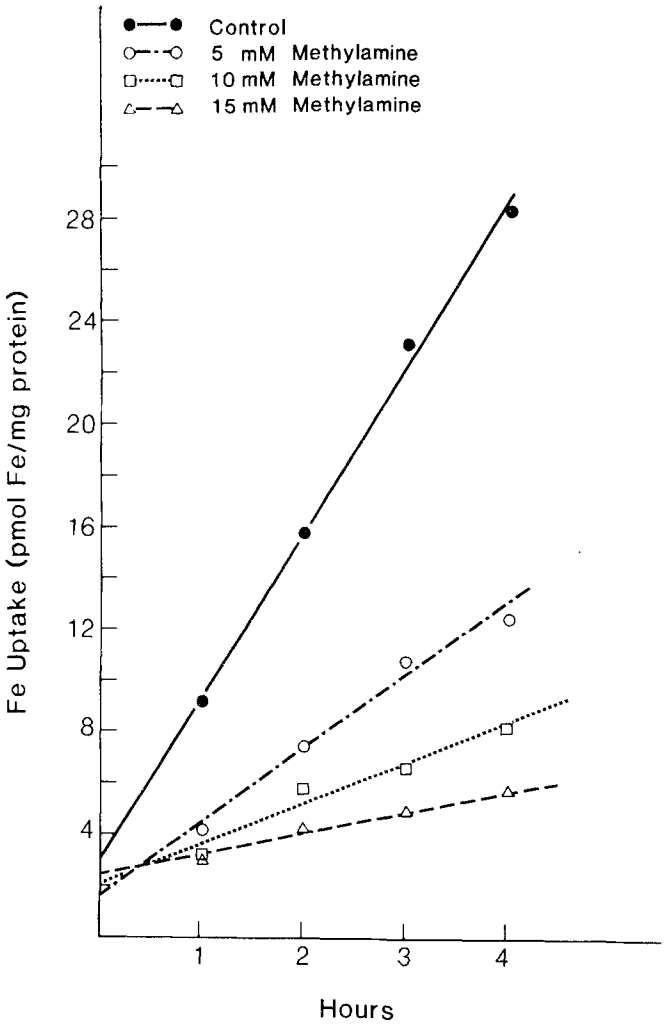

FIG. 2. Iron uptake into the free iron fraction was rapid and linear over 4 hours. Inhibition of iron uptake was linear at each concentration of CH_3NH_2. SEM ranged from 4%–8%.

DISCUSSION

The most salient biologic characteristic of iron is its capacity to support electron exchange and facilitate oxidation-reduction reactions. The oxidative capability of iron is also responsible for the toxic sequelae asso-

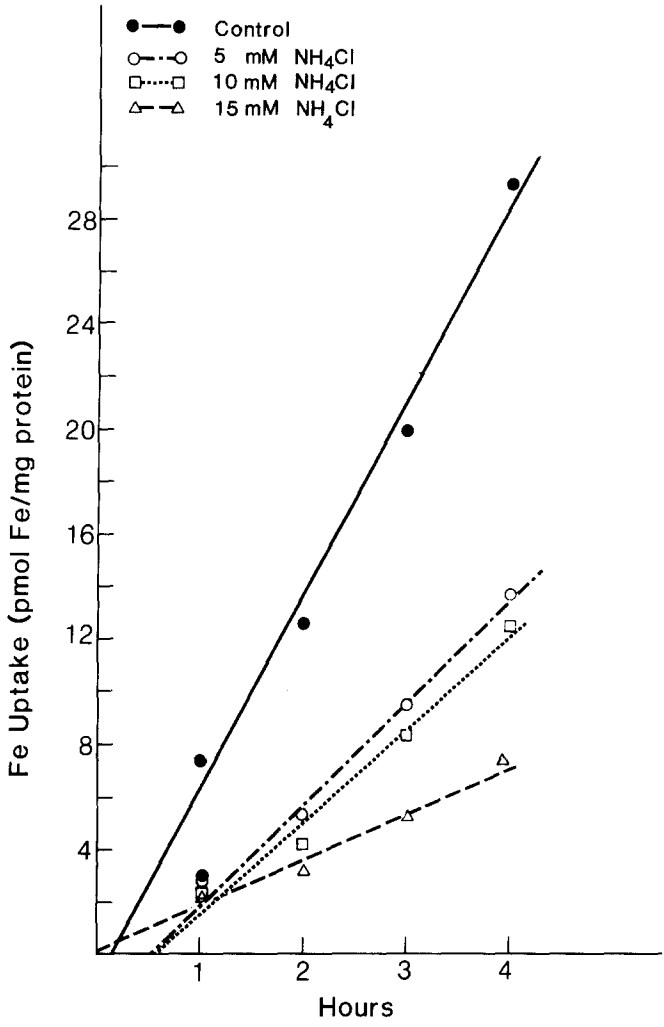

FIG. 3. Iron uptake into the free iron fraction was rapid and linear over 4 hours. Inhibition of iron uptake was linear at each concentration of NH_4Cl. SEM ranged from 4%–8%.

ciated with iron accumulation so that the ferric form may be responsible for most or all of the pathologic sequelae. Iron transport protein (e.g., transferrin) and iron storage protein (e.g., ferritin) are specially adapted to maintain metabolically available ferric iron concentration at negligible levels; however, this protective system is imperfect. Iron accumulation facilitates peroxidation of membranous debris with resultant lipofuscin

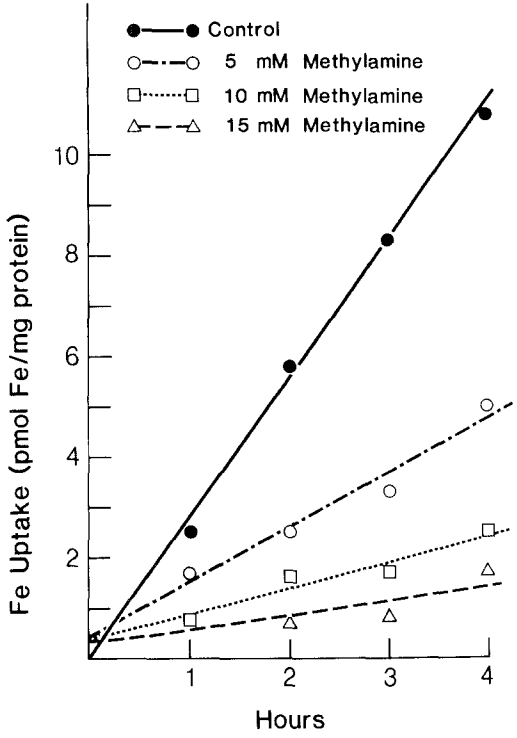

FIG. 4. Iron uptake into the ferritin-iron fraction was also rapid over the 4 hour period. CH_3NH_2 inhibition was evident and apparently linked to inhibition of iron uptake into the free iron fraction. SEM ranged from 7%–12%.

formation; subsequently the formation of neuromelanin as well as spheroid bodies occurs (26–28).

Brain areas which normally contain iron include (in order of highest to lowest concentration): globus pallidus, red nucleus, substantia nigra, putamen, dentate nucleus, caudate nucleus, motor cortex, thalamus, occipital cortex, and frontal white matter (10). In pathologic conditions iron deposits are often found in small cerebral blood vessels (e.g., Hallervorden-Spatz syndrome, syphilis, and Parkinson disease). A quantitative assessment of intracellular iron (e.g., neuronal or glial) in normal human brain or in conditions associated with increased iron storage is not available. Histochemical reports suggest that increased iron storage is rarely restricted to neurons. In some conditions iron storage occurs primarily in glial cells (e.g., neurosyphilis (16)); Pick disease (14); in other conditions increased iron stores are found in both glial and neuronal cells (e.g., Hal-

lervorden-Spatz syndrome (29) Parkinson disease (12), and Alzheimer disease (13)).

Transferrin (and associated iron) combines with its specific receptor on the plasma membrane surface (30). The resultant endosome transports the transferrin-receptor complex to the lysosome where the acidic milieu of the lysosome interior causes the transferrin-associated iron to be released. Alternate possibilities exist; for example, the transferrin-associated iron may be dissociated in the endosome before being incorporated in the lysosome (31). The iron-free transferrin (apotransferrin) is returned to the plasma membrane and eventually released from the cell. Intracellular iron is held in two relatively large, active metabolic compartments, ferritin-bound and free; generally the former is twice as large as the latter.

CH_3NH_2 and NH_4Cl are lysosomotropic and increase the pH of the lysosome cytosol thus discouraging deposition of materials including iron in lysosomes. CH_3NH_2 may also affect transferrin endocytosis by a similar mechanism (32).

It has been demonstrated previously that transferrin-iron uptake by fibroblasts is linear over 24 hours; it is inhibited by a number of substances including CH_3NH_2 (32). The studies reported here indicate that glial iron uptake is rapid and linear over 4 hours, and takes place both in the free and ferritin-bound fractions. Iron blocker studies demonstrate that NH_4Cl and CH_3NH_2 also inhibit iron uptake in glial cells much the same as they did in free reticulocytes and fibroblasts in other experimental systems (23, 32). Although uptake was generally similar to that of murine cortical neurons (1), important differences are apparent. Comparison of the data in the two experiments reveals that the total iron uptake by glial cells is greater than uptake by neuronal cells at 4 hours ($P < 0.005$). The degree of inhibition of total iron uptake by CH_3NH_2 (5 mM, 10 mM, and 15 mM) at 4 hours is also greater in glial cells ($P < 0.005$, $P < 0.001$, and $P < 0.001$ respectively). Although inhibition is present, the relative NH_4Cl inhibition of total iron uptake by glial or neuronal cells after 4 hours reveals no statistical differences.

Iron uptake into ferritin after 4 hours is also statistically greater in glial cells ($P < 0.005$). Similar comparison of the inhibitory effect of CH_3NH_2 (5 mM, 10 mM, and 15 mM) on iron uptake into ferritin in glial or neuronal cell cultures after 4 hours reveals no statistical difference at 5 mM, but statistically greater inhibition in glial cell cultures at 10 mM and 15 mM.

The data from these experiments demonstrate the likelihood that iron transport to glial cells is transferrin-mediated and the pattern generally parallels characteristics of uptake in other tissues which have been more

extensively studied. More refined studies are necessary to characterize nervous tissue iron transport and storage.

ACKNOWLEDGMENTS

We wish to thank Dr. Ruth Loewenson who provided statistical advice and analysis. The authors also wish to express their appreciation for the financial support of the American Parkinson Disease Association, The Children's Neuromovement Disorder Fund, and the Minnesota Medical Foundation.

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