

Identification of a Novel Route of Iron Transcytosis across the Mammalian Blood–Brain Barrier

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ABSTRACT

Objective: This study was undertaken to assess the role of p97 (also known as melanotransferrin) in the transfer of iron into the brain, because the passage of most large molecules is limited by the presence of the blood–brain barrier, including that of the serum iron transporter transferrin.

Methods: To study the function of the soluble form of p97, we followed the uptake of radioiodinated and ⁵⁵Fe loaded p97 and transferrin by the brain during a 24-hour period.

Results: We show that the soluble form of p97 has the ability to transcytose across the murine blood–brain barrier, and its transcytosis can be inhibited in a specific manner. We also provide evidence that p97 transports iron into the brain more efficiently than transferrin.

Conclusions: These data support the idea that p97 is an important iron transporter across the blood–brain barrier in normal physiology and possibly in neurodegenerative diseases, such as Alzheimer disease, in which iron homeostasis in the brain becomes disrupted.

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KEY WORDS: P97, melanotransferrin, transferrin, iron transport, brain, blood–brain barrier

INTRODUCTION

The blood–brain barrier (BBB) is formed by specialized capillary endothelial cells that act as a selective barrier between the peripheral blood and the brain tissues. This barrier limits the passive diffusion of many molecules, such as hormones and ions, which act to maintain the unique environment of the brain.

Although the presence of specific transport systems within the endothelial cells must ensure that the brain receives all necessary compounds, the mechanisms of selective trafficking of macromolecules and peptides through the BBB are not well understood.

One essential element for cellular metabolism is iron. Free iron is cytotoxic, however, and its level is tightly regulated in normal physiology. Abnormally high levels of iron leading to free radical formation have been demonstrated in various neurodegenerative diseases, including Alzheimer disease (AD) (13,24) and cancer (32). Mechanisms that underlie iron uptake into the brain have remained a central mystery in brain physiology. Because transferrin (Tf) is abundant in the blood and the classically defined Tf and transferrin receptor (TfR) mediate iron uptake

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into most cells, it was assumed that iron uptake by the brain is also carried out by the same system. Several studies, however, have come to challenge the exclusive role of Tf in brain iron transport.

Since TfR was first localized on cerebral endothelia in adult rats and humans (12), its participation in iron transport across the BBB has not been questioned. Further studies have confirmed the localization of TfR on the BBB (18,28), and monoclonal antibodies against TfR were shown to cross the BBB (16,21). In contrast, the brain distribution of Tf, the main ligand for TfR, is surprisingly limited to oligodendrocytes and not to endothelia (4,19). Furthermore, studies have shown that the activity of Tf alone cannot account for the iron accumulated in the brain (20,27). As an explanation, researchers suggest that Tf releases iron in the cytosol of cerebral endothelia (7,25,26). The iron released in the cytoplasm of the endothelial cell, in turn, might then become bound to endogenously expressed brain Tf, whereas the serum Tf transported into endothelia is recycled out of the brain. This was demonstrated using a perfusion model, in which only a small fraction of total Tf injected into mice transcytosed into the brain, whereas much of it was retained in the endothelia as an intracellular pool (7). More importantly, other studies have shown that hypotransferrinaemic mice deficient in Tf have a higher level of iron uptake by the brain than normal mice (6,31), suggesting that the current description of the Tf/TfR model for brain iron uptake is at the very least incomplete.

The disparate conclusions drawn from these studies could be reconciled if other modes of iron transcytosis across the BBB exist. We focused on another iron-carrying protein, p97 (also known as melanotransferrin), because of its colocalization with the TfR on the BBB (28), and studied its potential role for iron delivery into the brain. p97 belongs to a family of iron-binding proteins that include serum Tf, lactoferrin, and ovotransferrin. Human p97 shares 39% sequence identity with human Tf (23) and has been shown to bind iron (1,14). Unlike other Tf family members, this molecule exists in two forms, a glycosyl-phosphatidylinositol (GPI)-linked cell surface form and a secreted soluble form generated by alternative splicing (8,11,17). The level of soluble p97 is elevated in the serum of patients with AD, suggesting that the protein might contribute to the pronounced accumulation of iron in the brains of patients with AD (14,15). To further understand the mechanisms of iron uptake by the brain, we exam-

ined the ability of soluble p97 to cross the BBB *in vivo*.

MATERIALS AND METHODS

Protein Preparation

Soluble human p97 was obtained by concentrating and purifying soluble p97 transfected baby hamster kidney cell supernatant as described (33). Soluble p97 was first dialyzed to remove any bound iron before the experiment. Iron was loaded to p97 and mouse Tf by adding 10 nM of soluble apo-p97 to 10 mM of iron nitrilotriacetate (FeNTA) in the presence of 250 mM sodium bicarbonate. All experiments used human holo-p97 and mouse holo-Tf with the exception of microscopic examination in which human holo-p97 and human holo-Tf were used to distinguish the injected proteins from endogenous mouse proteins. For iodination, human p97, mouse Tf (Sigma, Oakville, Ontario, Canada), and bovine serum albumin (BSA; Sigma) were labeled using the chloramine T method and purified with Sephadex G25 columns (Pharmacia, Baie d'Urfé, PQ, Canada). Specific activities of ^{125}I -p97 and ^{125}I -Tf were determined to be 6×10^{18} cpm/mole and 9×10^{18} cpm/mole, respectively. Trichloroacetic acid precipitability was approximately 95%. For loading ^{55}Fe to proteins, 30 μL of 5 mM $^{55}\text{FeCl}_3$ (NEN, Boston, MA) was added to 60 μL of 25 mM sodium citrate. The solution was neutralized with 24 μL of 1 M sodium bicarbonate and 2.5 mg of human p97 or mouse Tf was added for 1 hour at 37 °C. The ^{55}Fe -loaded proteins were dialyzed in PBS twice to remove unbound ^{55}Fe citrate. Specific activities of ^{55}Fe -p97 and ^{55}Fe -Tf were 7.4×10^{14} dpm/mole and 1.9×10^{15} dpm/mole, respectively.

Transcytosis of p97 and Tf Across the BBB

For the ^{125}I -protein uptake time course and dose response experiment, 5.5×10^{-14} moles/g body mass of ^{125}I -human p97 or ^{125}I -mouse Tf was injected into the tail vein of C57Bl/6 mice (Fig. 1). In the competition study, 100 times concentration of cold human p97 or mouse Tf were added with the 5×10^{-14} moles ^{125}I -p97/g body mass and injected into the tail vein. For the time course of iron uptake into the brain, 2.5×10^{-11} moles/g body mass of ^{55}Fe -p97 or 1×10^{-11} moles/g body mass of ^{55}Fe -Tf was injected into the tail vein (Fig. 4a-c). After 1, 3, 6, or 24 hours, the mice were anesthetized with ketamine and xylazine. After a blood sample was collected from the right atrium, the mice were perfused with PBS containing 0.1% BSA through the left ventricle of the heart. Tissues were then collected and weighed, and radioactivity was determined using ei-

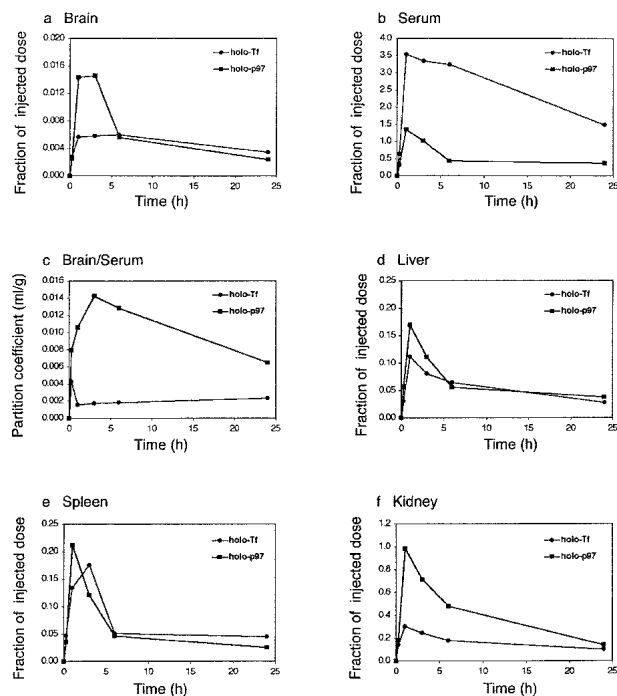


Figure 1. Uptake of p97 and Tf in vivo. The activity of ^{125}I -p97 (solid square) and ^{125}I -Tf (solid circle) in various tissues during a 24-hour period expressed as mass specific fraction of injected dose (g tissue/g body mass). (a) brain, (b) serum, (c) brain/serum partition coefficient (mL/g), (d) liver, (e) spleen, (f) kidney.

ther a gamma or scintillation counter. Blood samples were centrifuged at 11,000 rpm at 4 °C, the pellet was discarded, and the serum was collected for counting. The brain was capillary depleted as described by Triguero et al. (30). After dextran fractionation, light microscopy examination showed that the pellet consisted mostly of brain vasculature, whereas the top layer was essentially devoid of brain vasculature. In another set of experiments, 2.5×10^{-10} moles/g body mass of ^{55}Fe -p97 or 1×10^{-10} moles/g body mass of ^{55}Fe -Tf or 1.9×10^5 dpm ^{55}Fe -citrate/g body mass was injected into the tail vein of mice (eight mice per chelate). One hour after injection, the mice were killed, and venous blood samples were taken for analysis. After perfusion, the brain was removed and processed whole for the measurement of radioactivity by liquid scintillation counting. The data were analyzed by ANOVA. All data are expressed as mass specific fraction of injected dose ([activity/g tissue]/[activity of injected dose/g body mass]).

Protein Analysis

To demonstrate that some iodinated holo-p97 crossing into the brain in the transecytosis studies re-

mained intact, the mice were perfused with PBS 1 hour after 6×10^6 cpm of ^{125}I -p97 was injected. Capillary depleted brain parenchyma was lysed in 1% Triton X-100 in 20 mM Tris-Cl, 2 mM EDTA, and 150 mM NaCl (pH 7.5) for 30 minutes at 4 °C. The lysate was collected and centrifuged at 11,000 rpm at 4 °C for 10 minutes. Because the level of radioactivity in the parenchyma after capillary depletion was too low to be detected using autoradiography, an immunoprecipitation using the monoclonal anti-p97 antibody L235 (ATCC, Manassas, VA) and Western blot was carried out for these samples as previously described (8).

RESULTS

Uptake of ^{125}I -p97 and ^{125}I -Tf by the Brain

Time course studies show that the initial amount of ^{125}I -p97 taken up by the brain rises sharply, reaching a peak of approximately 0.015 of injected dose within 1 hour of injection (Fig. 1a). Most ^{125}I -p97 is cleared from the brain within 5 hours, and the level returns to baseline in 24 hours. In comparison, the uptake of ^{125}I -Tf by the brain reaches a smaller plateau (0.005 of injected dose) 1 hour after injection and is gradually cleared during 24 hours. This is in sharp contrast to the profile in serum in which ^{125}I -Tf reaches a peak that is 2.6-fold greater than the ^{125}I -p97 peak. Furthermore, ^{125}I -p97 in the serum is cleared within 5 hours, whereas ^{125}I -Tf remains in the serum for up to 24 hours after injection. The brain to serum partition coefficient (mL/g) for ^{125}I -p97 is sevenfold higher than for ^{125}I -Tf, indicating preferential uptake of p97 by the brain (Fig. 1c). In contrast to the uptake by the brain, ^{125}I -p97 and ^{125}I -Tf are quickly incorporated into the liver and spleen reaching comparative peaks and are cleared within 5 hours of injection (Fig. 1d and e). A different pattern was noted for kidney, in which ^{125}I -p97 uptake was approximately threefold greater than ^{125}I -Tf (Fig. 1f).

To show that p97 that crosses the BBB into the brain remains intact, mice were injected with p97, and proteins were recovered from the parenchyma after perfusion and capillary depletion (30). Immunoprecipitation of the mouse parenchyma demonstrate a band at 97 kDa, suggesting that a significant portion of the injected p97 remained intact 1 hour after injection (Fig. 2).

The ability of unlabeled p97 and/or Tf to inhibit ^{125}I -p97 uptake into the brain was examined to establish the specificity of this process. The data in Fig. 3 show that the uptake of ^{125}I -holo-p97 is

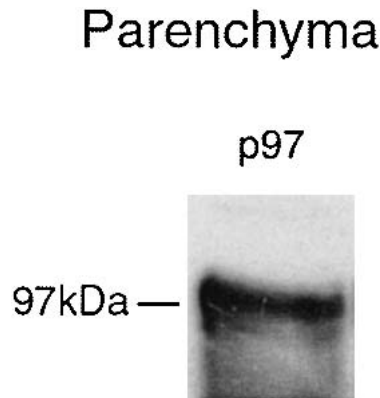


Figure 2. Recovery of p97 from the brain. Immunoprecipitation of p97 from parenchyma harvested 1 hour after injection, detected by chemiluminescence on Western blot.

partly inhibited by excess cold p97, whereas p97 uptake is less affected by the equivalent dose of cold Tf. The addition of cold p97 rapidly inhibits ^{125}I -p97 uptake and seems to cause its clearance from the system. Introduction of cold Tf, on the other hand, inhibits the uptake of ^{125}I -p97 in a less-specific manner than cold p97 and further reduces the clearance rate from the brain.

Uptake of ^{55}Fe -loaded p97 and ^{55}Fe -loaded Tf by the Brain

Efficiency of p97 and Tf to iron transport through the BBB into the mouse brain was examined by injecting ^{55}Fe -loaded p97 or Tf in vivo (Fig. 4). After 1 hour, iron bound to p97 accumulated in the brain parenchyma and vasculature 6 and 10 times more than iron bound to transferrin, respectively. The results also show that p97 in the parenchymal fraction are higher than that of the endothelial fraction. The brain-to-serum partition coefficient for ^{55}Fe -p97 is eight to nine times higher than ^{55}Fe -Tf 1 to 6 hours after injection (Fig. 4c, d). Interestingly, after the initial peak, only a slight uptake of ^{55}Fe is observed in the brain by either p97 or Tf.

DISCUSSION

We have used a combination of physiologic and cellular techniques to experimentally address the hypothesis that the p97 molecule is capable of crossing the BBB and that it is a major mediator shuttling iron into the brain. The ability of p97 to transcytose the BBB in vivo was examined first (Fig. 1). Our data show that p97 can cross the BBB in its intact form within 1 hour of injection and is largely catabolized

and eliminated from brain tissues within 5 hours (Figs. 1 and 2). Soluble p97 transcytosed through the BBB into the brain more readily than Tf, even though the level of Tf in serum remained consistently higher than p97. The level of p97 uptake was higher than Tf in the kidney, whereas in the liver and spleen, comparative uptake profiles of p97 and Tf were recorded. Taken together, these results indicate that a large fraction of Tf is not likely to be taken up by the brain under normal conditions. This is consistent with several studies indicating that serum Tf is not the exclusive carrier of iron into the brain (7,20,27). It should be noted that in this study we used the available soluble human p97 compared with mouse Tf. It is likely that the murine homologue of p97, when it becomes available, should perform as well or better than the human orthologue.

In addition, we show that cold p97 seems to inhibit ^{125}I -p97 uptake with higher specificity than cold Tf using an in vivo competition assay (Fig. 3). Collectively, these results suggest p97 could interact with a receptor or receptors in vivo. Recently, low-density lipoprotein receptor has been suggested as a possible receptor for p97 (5). Low-density lipoprotein receptor, however, interacts with a large number of ligands, and it is possible that other receptors exist, which are more specific for p97 binding. Further study is needed to link ligand-receptor interaction to receptor localization on cerebral endothelial cells.

There is growing interest in designing molecules that can be transported into the brain by means of specific receptors naturally expressed in brain capillary

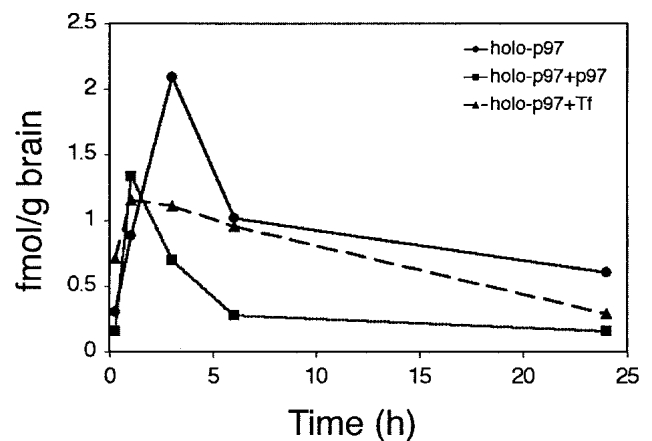


Figure 3. Uptake of ^{125}I -p97 is inhibited by p97 or Tf. The uptake of ^{125}I -p97 by brain tissue (circle) is inhibited more by the addition of 100 \times more cold p97 (square) than by the addition of an equal amount of cold Tf (triangle) during a 24-hour period.

endothelium. Some of the candidates for drug targeting to the brain include antibodies against TfR (16,21), glucose transporter (9,10), and a HIV gp120-derived peptide (2,3). The use of these molecules and others as carriers across the BBB might allow less-invasive and more-efficient delivery of therapeutic agents to the brain for the treatment of various neurodegenerative diseases and tumors. Because p97 seems to cross the BBB, it might act as an efficient carrier of therapeutic compounds and genes into the brain. Because p97 is a natural protein in human, it is unlikely to stimulate an immune response. Furthermore, the relatively rapid turnover rate of p97 in vivo compared with some antibodies suggests that p97 might be used for repetitive injection of conjugates.

After showing that p97 crosses the BBB from blood into brain parenchyma in vivo, we directly wanted to address whether p97 could mediate iron transport to the brain. The ability of p97 to bind iron has been established previously (1) and is also visible on a urea gel similar to Tf. The uptake of ^{55}Fe by the brain mediated by p97 was approximately eight to nine times higher than that of Tf after 1 to 6 hours after injection (Fig. 4).

Elevated levels of free iron are noted in various neurodegenerative diseases, including AD (24). It is well

noted that the formation of free radicals resulting from excessive accumulation of free iron is particularly damaging to the sensitive tissues of the central nervous system (29). In addition to AD, excessive iron deposition and oxidative stress is associated with the pathologies of a number of neurodegenerative disorders, including aceruloplasminemia (34) and progressive supranuclear palsy (22). In addition to its potential usefulness as a drug shuttling molecule, p97 could play a central role in modulating iron transport in AD patients, because tantalizing studies have shown that the levels of p97 are elevated in the serum (14,15) and cerebrospinal fluid (14). Thus, the identity of the protein carrier that shuttles iron into the brain might be at last resolved, revealing a function for the secreted form of p97. Further investigations are being carried out to identify potential receptor(s), modes of transcytosis, and destinations of p97.

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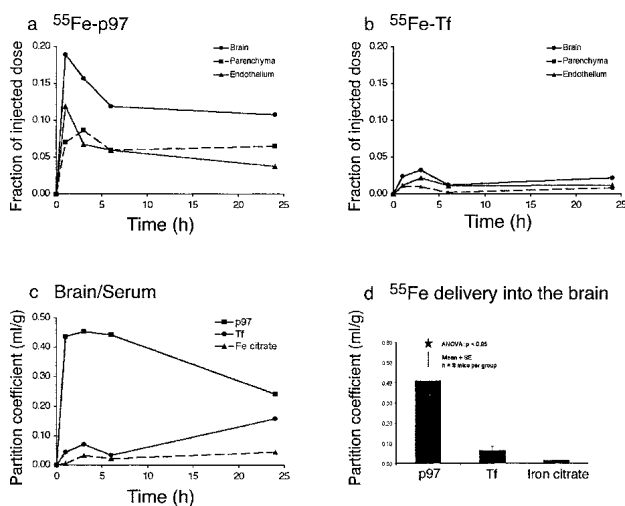


Figure 4. Iron transport into the brain. (a) ^{55}Fe transport into the entire brain (circle), brain parenchyma (square), and brain endovasculture (triangle) mediated by (a) p97 or (b) Tf. The brain/serum partition coefficient (c) for ^{55}Fe transport is compared between p97 (square), Tf (circle), and Fe-citrate (triangle) during a 24-hour period. Statistical analysis (ANOVA) of ^{55}Fe transport into the brain 1 hour after injection of equivalent amounts of ^{55}Fe -p97, ^{55}Fe -Tf, or ^{55}Fe -citrate (d).

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