Pharmaceutical Research, Vol. 21, No. 5, May 2004 (© 2004) *Research Paper*

Characterization and Modulation of the Transferrin Receptor on Brain Capillary Endothelial Cells

Corine C. Visser,[1] L. Heleen Voorwinden,[1] Daan J. A. Crommelin,[2] Meindert Danhof,[1] and Albertus G. de Boer[1,3]

Received November 17, 2003; accepted February 9, 2004

Purpose. The expression level of the transferrin receptor (TfR) on brain capillary endothelial cells (BCECs) and the endocytosis of ^{125}I-transferrin (^{125}I-Tf) by this receptor was investigated. Furthermore, the influence of iron, the iron scavenger deferoxamine mesylate (DFO), astrocytic factors, a GTP-ase inhibitor (tyrphostin-A8, T8), lipopolysaccharide (LPS), and the radical scavenger *N*-acetyl-L-cysteine (NAC) on the TfR expression was studied to gain insight in the use and optimization of the TfR for drug targeting to the brain.
Methods. Experiments were performed with primary cultured bovine BCECs that were incubated with ^{125}I-Tf at 4°C (to determine binding) or at 37°C (to determine endocytosis) in the absence or presence of the modulators. For full saturation curves in the absence or presence of iron or DFO, analysis was performed with a population approach using NONMEM, allowing us to estimate a single value for affinity (K_d, concentration of 50% receptor occupancy) and separate values for maximum receptor occupancy (B_{max}).
Results. On BCECs, the TfR is expressed extracellularly (B_{max} of 0.13 fmol/μg cell protein), but also has a large intracellular pool (total B_{max} of 1.37 fmol/μg cell protein), and is actively endocytosing Tf via clathrin-coated vesicles. At 4°C, a K_d of 2.38 μg/ml was found, whereas the K_d at 37°C was 5.03 μg/ml. Furthermore, DFO is able to increase both the extracellular as well as the total binding capacity to 0.63 and 3.67 fmol/μg cell protein, respectively, whereas it had no influence on K_d. B_{max} at 37°C after DFO preincubation was also increased from 0.90 to 2.31 fmol/μg cell protein. Other modulators had no significant influence on the TfR expression levels, though LPS increased cellular protein concentrations after 2-h preincubation.
Conclusions. The TfR is expressed on BCECs and actively endocytoses Tf, making it a suitable target for drug delivery to the blood-brain barrier and the CNS. DFO up-regulates the TfR expression level, which may influence targeting efficiency.

KEY WORDS: astrocytic factors; deferoxamine; drug targeting; *in vitro* blood-brain barrier; iron; lipopolysaccharide; modulation; *N*-acetyl-L-cysteine; receptor-mediated endocytosis; tyrphostin-A8.

[1] Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Division of Pharmacology, 2300 RA Leiden, The Netherlands.
[2] Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), 3508 TB Utrecht, The Netherlands.
[3] To whom correspondence should be addressed. (e-mail: b.boer@lacdr.leidenuniv.nl)

ABBREVIATIONS: BCECs, brain capillary endothelial cells; DFO, deferoxamine mesylate; LPS, lipopolysaccharide; NAC, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide; PhAsO, phenylarsine oxide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; Tf, holo-transferrin; TfR, transferrin receptor; T-8, Tyrphostin-A8.

INTRODUCTION

The central nervous system (CNS) is protected by the blood-brain barrier (BBB) to maintain homeostasis. This barrier is situated at the brain capillaries and comprises endothelial cells, covered by the endfeet of astrocytes (1). Due to specific features, such as tight junctions between endothelial cells, a continuous basal membrane, low pinocytosis, and a lack of fenestrae, in general only small lipophilic drugs can pass the BBB (2). Many drugs for disorders of the CNS do not meet these requirements. Therefore, special transport systems are necessary to transport these drugs to the brain (3,4).

To avoid invasive strategies to enhance BBB permeability, such as osmotic BBB disruption, targeting to the CNS often is aimed at endogenous transporters (5), such as the insulin receptor (6), the LDL receptor (7), or the scavenger and HDL receptor (8). Our research focuses on the use of the transferrin receptor (TfR), which is an internalizing receptor, for brain drug targeting. It has been shown that drugs targeted to this receptor with a monoclonal antibody-conjugate have an enhanced biological effect in the brain *in vivo* (9). The TfR is expressed on endothelial cells of the brain capillaries where it is involved in iron transport to the brain via receptor-mediated endocytosis of transferrin (Tf) (10). Furthermore, the TfR is also expressed on hepatocytes, erythrocytes, and on proliferating cells (11). The TfR is a 190-kDa transmembrane glycoprotein, consisting of two subunits that are linked by a disulfide bridge (10). A trypsin-sensitive site is present extracellularly, and proteolytic cleavage at this site leads to the loss of Tf binding (11). Recently, a second TfR has been identified, TfR2 (12). TfR2 also delivers iron to cells, but it has a 25 times lower affinity for Tf, and the distribution of TfR2 is different from TfR. Our research focuses on the TfR.

The objective of this research is to characterize the TfR mechanistically, in the *in vitro* BBB model developed by Gaillard *et al.* (13), for studying brain drug targeting to and through this receptor. Therefore, we have done extensive studies; not only to determine binding and association (i.e., a combination of binding and endocytosis), but also the extent and mechanism of endocytosis. Furthermore, the influence of several modulators on the TfR expression was studied to gain insight into the potential use of the TfR for drug targeting to the brain.

First, the binding characteristics and association of Tf by the TfR at the BBB *in vitro* were investigated, as well as the extent of endocytosis of Tf. The latter was done by removal of extracellularly bound ^{125}I-Tf by acid wash or proteolytic cleavage of the TfR. In addition, to investigate the mechanism of endocytosis, the influence of several inhibitors of endocytotic processes was studied. For this, phenylarsine oxide (PhAsO) was used, as it inhibits the clathrin-associated receptor-mediated pathway, which is associated with the TfR (14). *N*-ethylmaleimide (NEM) is used as a nonspecific inhibitor (15) and indomethacin as an inhibitor of adsorptive-mediated endocytosis, associated with caveolae (15).

Subsequently, changes in TfR expression, following preincubation with several modulators, were studied. First, the influence of iron on the binding and association of ^{125}I-Tf was investigated. The expression level of the TfR is mainly dependent on the iron concentration, as the mRNA of the TfR is stabilized by an iron-regulatory factor at low iron concen-

0724-8741/04/0500-0761/0 © 2004 Plenum Publishing Corporation

trations, but not at high concentrations (16,17). Therefore, the TfR expression level was determined at high iron concentrations (18) by addition of an excess of $FeCl_3$, and at low iron concentrations by addition of the iron scavenger deferroxamine mesylate (DFO) (19). Furthermore, the influence of astrocytes on TfR expression and association was estimated, as it is known that the secretion of astrocytic factors induce and maintain many BBB properties of the brain capillary endothelial cells (BCECs) (2). For the purpose of validating BBB or brain drug targeting models, it was highly relevant to determine the level of TfR expression in the absence or presence of astrocytic factors. Recently, it was shown that the GTP-ase inhibitor tyrphostin-A8 (T8) can increase the transcytosis of Tf-conjugates in Caco-2 cells (20). Therefore, we investigated the influence of T8 on the binding and endocytosis of ^{125}I-Tf. Finally, BCECs were stimulated with lipopolysaccharide (LPS) to study TfR expression under inflammatory disease conditions. The effect of LPS was determined after a short-time (2 h) incubation, during which acute phase effects occur, and after 24-h incubation, to assess the long-term effects. In addition, BCECs were preincubated with the radical scavenger *N*-acetyl-L-cystein (NAC), as it was shown that NAC could prevent the down-regulation of TfR expression by free radicals (21).

MATERIALS AND METHODS

Materials

Culture flasks were obtained from Greiner (Alphen a/d Rijn, The Netherlands) and 48-well plates from Corning Costar (Cambridge, MA, USA). PBS, DMEM, supplements, and fetal calf serum were purchased from BioWhittaker Europe (Verviers, Belgium). Type IV collagen, heparin, trypsin-EDTA, endothelial cell trypsin, Iodogen™, saponin, phenylmethylsulfonylfluoride (PMSF), NEM, PhAsO, indomethacin, LPS, DFO, and $FeCl_3{\cdot}6\ H_20$ were obtained from Sigma (Zwijndrecht, The Netherlands), fibronectin from Boehringer Mannheim (Almere, The Netherlands), and leupeptin from Molecular Probes (Leiden, The Netherlands). Bovine holo-transferrin, T8, and NAC were purchased from ICN Pharmaceuticals (Zoetermeer, The Netherlands) and Bio-Rad DC protein assay reagents from Bio-Rad Laboratories (Veenendaal, The Netherlands). Citric acid monohydrate and trichloricacetic acid (TCA) were obtained from J. T. Baker (Deventer, The Netherlands) and di-sodium hydrogen phosphate dihydrate from Merck (Amsterdam, The Netherlands). Sephadex-G25 coarse and [^{125}I]Na were purchased from Amersham Pharmacia Biotech (Roosendaal, The Netherlands).

Cell Culture

Primary brain capillary endothelial cells (BCEC) were cultured from isolated bovine brain capillaries as previously described (13). Briefly, brain capillaries were seeded in type IV collagen and fibronectin-coated plastic culture flasks and cultured in a 1:1 mixture of DMEM+S (containing 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids, and 10% fetal calf serum) and astrocyte-conditioned medium (ACM), supplemented with 125 μg/ml heparin (DMEM+ACM) at 37°C, 10% CO_2 for 4–5 days. At 70% confluency, the BCECs were passaged with trypsin-EDTA and seeded into a type IV collagen-coated 48-well plate at a density of 30,000 cells/well. BCECs were cultured in the same medium at 37°C, 10% CO_2 for 5 days.

Astrocyte-conditioned medium was obtained according to the method described by Gaillard *et al.* (13).

Preparation of Radiolabeled Transferrin

Bovine Tf was iodinated using Iodogen™, as previously described (22), with a few modifications. Two hundred micrograms of Tf (2.5 mg/ml in 1.5 M Tris-HCl, pH 8.5) was added to 0.25 mCi [^{125}I]Na in an Iodogen™ (10 μg) precoated tube and incubated for 30 min at 4°C. After separation on a Sephadex G-25 column, the labeled Tf was further purified by extensive dialysis (at least 48 h, 4 changes of buffer) in phosphate-buffered saline (pH 7.4) at 4°C. The labeled Tf had a specific activity of $278 \pm 199 \times 10^3$ cpm/μg and contained <3% free ^{125}I (determined by precipitation with 10% (w/v) trichloroacetic acid). ^{125}I-Tf was stored at 4°C and used within 2 weeks.

Determination of Cell-Associated Transferrin (General)

BCECs were checked under the microscope for confluency and morphology (spindle shape when confluent) (13). One hour prior to the experiment, the medium was changed to DMEM to deplete the cells from endogenous Tf. Subsequently, BCECs were incubated with ^{125}I-Tf in a concentration range of 0.25–12 μg/ml (full saturation approach), or with a fixed concentration of 8 μg/ml ^{125}I-Tf in 100 μl PBS. Incubation was performed at 4°C (to determine binding) for 2 h or at 37°C (to determine association, which is a combination of binding and endocytosis) for 1 h. For the determination of the total receptor expression level, 0.5% (w/v) saponin, 1 mM PMSF, and 1 μg/ml leupeptin were added to permeabilize the cellular membranes (23). After incubation, BCECs were washed 6 times with 0.5 ml ice-cold PBS and solubilized with 1 M NaOH (750 μl) to measure radioactivity (gamma-counter) or the cellular protein content (Bio-Rad DC protein assay).

Endocytosis Experiments

To determine the extent of endocytosis, BCECs were incubated with 8 μg/ml ^{125}I-Tf in 100 μl PBS at 4°C for 2 h or at 37°C for 1 h and rinsed twice with 0.5 ml ice-cold PBS. Thereafter, BCECs were incubated with 0.5 ml citric acid/phosphate buffer pH 5.0 (modification of Ref. 24) for 10 min on ice or with 0.5 ml trypsin (0.25 mg/ml) for 30 min on ice (25). After acid wash, cells were quickly washed twice with citric acid/phosphate buffer and three times with PBS before solubilization with NaOH. After trypsinization, cells were transferred to a tube containing DMEM with 10% fetal calf serum and centrifuged for 5 min at 400*g*. BCECs were washed twice with PBS before determination of the remaining cell-associated activity.

For the inhibition studies, BCECs were preincubated for 10 min with PhAsO (10 μM), NEM (1 mM), or indomethacin (50 μg/ml). Concentrations and preincubation times were modified from literature (14,15). Subsequently, BCECs were incubated with 8 μg/ml ^{125}I-Tf in 100 μl PBS in the presence of the inhibitors at 4°C for 2 h or at 37°C for 1 h. BCECs were washed and solubilized as previously described.

Modulation by Iron, Astrocytic Factors, and Tyrphostin A8

To determine the influence of iron, BCECs were preincubated for 24 h with 1 mM DFO or $FeCl_3$ before estimating binding and association of ^{125}I-Tf using the full saturation approach. Association is a combination of binding and endocytosis, as we have not discriminated between those, unless specified.

To determine the influence of astrocytic factors on the TfR expression level, BCECs were cultured for 5 days in either the normal medium, which is a 1:1 mixture of DMEM+S and ACM, supplemented with 125 μg/ml heparin (DMEM+ACM), or in DMEM+S supplemented with 125 μg/ml heparin (DMEM+hep) or in DMEM+S alone (DMEM+S). Iron and Tf concentrations in ACM and DMEM+S were determined by a colorimetric assay on a fully automated Hitachi 911 (Hitachi, Tokyo, Japan). Coefficients of variation of these assays are below 3%.

To determine the influence of T8 on the endocytosis of Tf, BCECs were preincubated for 10 min with 0.125–0.5 mM T8. Subsequently, binding and association of ^{125}I-Tf were determined in the presence or absence of T8.

Inflammatory Disease Conditions

BCECs were preincubated with 100 ng/ml LPS for 2 or 24 h before the binding and association of ^{125}I-Tf were assessed. The effect of NAC was determined by a 1-h or an overnight (16–17 h) preincubation with 10 mM NAC, followed by a preincubation with LPS for 2 h. Concentrations are from Gaillard *et al.* (26), who showed that 100 ng/ml LPS increases the permeability of the BBB *in vitro* after 2 h. This effect was reversed by an overnight preincubation with 10 mM NAC.

Data Analysis

In all experiments, total binding was corrected for nonspecific binding, which was determined in the presence of 500-fold excess of unlabeled Tf. All data are presented as the means of at least three individual experiments, performed in triplicate. Cpm values were corrected for the specific activity of the batch of ^{125}I-Tf used for the experiment and for the cellular protein content.

Full saturation experiments were analyzed with a population approach using the conventional first-order method implemented in NONMEM (version V, NONMEM project group, University of California, San Francisco, CA, USA). A user-defined model for an one-site binding approach, where $B = B_{max}[C]/(K_d + [C])$, was implemented. In this equation, B is the specific binding, B_{max} the maximal receptor occupancy, C is the concentration, and K_d is the concentration at which 50% receptor occupancy occurs. By using this population approach, it was possible to estimate a single K_d value for all binding experiments and different B_{max} values for the total and extracellular expression level in control situation or after preincubation with DFO or $FeCl_3$. K_d and B_{max} values are estimated for both 4°C and 37°C. B_{max} values at 37°C represent the maximal receptor occupancy as a combination of binding and endocytosis. Intra-individual residual variation was determined using a proportional error model, and model selection was based on the parameter estimates, parameter correlations, and their confidence intervals. Goodness-of-fit was analyzed by visual inspection as well as by the minimum value of the objective function provided by NONMEM.

Statistical analysis was performed by one-way ANOVA (Tukey-Kramer multiple comparison post-test) and the Student's *t* test, using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA).

RESULTS

Full Saturation Binding Studies

TfR expression was determined by incubating the BCECs with 0.25–12 μg/ml ^{125}I-Tf for 2 h at 4°C. At a concentration of approximately 8 μg/ml, ^{125}I-Tf full saturation of the TfR expressed on BCECs was observed, as is shown in Figs. 1A and 1B. Using the population approach, a single unique value of the K_d was estimated, which was 2.38 ± 0.32 μg/ml, whereas separate B_{max} values were obtained for total and extracellular TfR expression. These were 1.37 ± 0.11 and 0.13 ± 0.02 fmol/μg protein (Table I), respectively, indicating that approximately 90% of the TfRs is present in a large intracellular pool. After incubation of BCECs with ^{125}I-Tf at 37°C, a K_d of 5.03 ± 0.50 μg/ml was found, whereas B_{max} at 37°C was estimated at 0.90 ± 0.06 fmol/μg protein (Table I).

After preincubation with 1 mM DFO, the total and the extracellular TfR expression were increased to 3.68 ± 0.48 and 0.63 ± 0.04 fmol/μg protein, respectively (Figs. 1A and 1B; Table I). Preincubation with $FeCl_3$ did not change the total TfR expression level (1.64 ± 0.28 fmol/μg protein), whereas the B_{max} for the extracellular TfR expression level was too low to detect.

Experiments at 37°C showed a twofold increase in B_{max} to 2.31 ± 0.14 fmol/μg protein after DFO preincubation, whereas after preincubation with $FeCl_3$, a B_{max} of 0.56 ± 0.04 fmol/μg protein was observed (Fig. 1C; Table I).

Endocytosis

To show that the TfR is actively endocytosing its ligand, extracellularly bound ligand was removed by either washing with a citric acid buffer or by removal of the extracellular part of the TfR by trypsin cleavage. At 37°C, 0.05–0.06 ng Tf/μg cell protein remained cell-associated after acid wash or trypsin cleavage (Fig. 2), which is 70–80% of the total amount of cell-associated Tf (0.07 ng Tf/μg cell protein). After binding of ^{125}I-Tf at 4°C and trypsin cleavage, the cell-associated Tf was of the level of nonspecific binding (0.003 ng Tf/μg cell protein, $p < 0.01$ vs. total), whereas after acid wash, approximately 50% of the extracellular bound Tf was still present (0.007 ng Tf/μg cell protein).

Several inhibitors were used to study the mechanism of the endocytotic process. BCECs were incubated with 8 μg/ml ^{125}I-Tf at 4°C for 2 h or at 37°C for 1 h in the absence or presence of PhAsO, an inhibitor of clathrin-mediated endocytosis, NEM, an inhibitor of most endocytotic processes, or indomethacin, an inhibitor of caveolae mediated transport (Fig. 3). Total cell-associated Tf was 0.07 ng/μg cell protein; PhAsO and NEM inhibited the endocytosis of ^{125}I-Tf almost completely to 0.003 and 0.007 ng Tf/μg cell protein, respectively. PhAsO and NEM had no effect on the binding of ^{125}I-Tf. Indomethacin had no significant effect on either the binding nor the endocytosis of ^{125}I-Tf (Fig. 3).

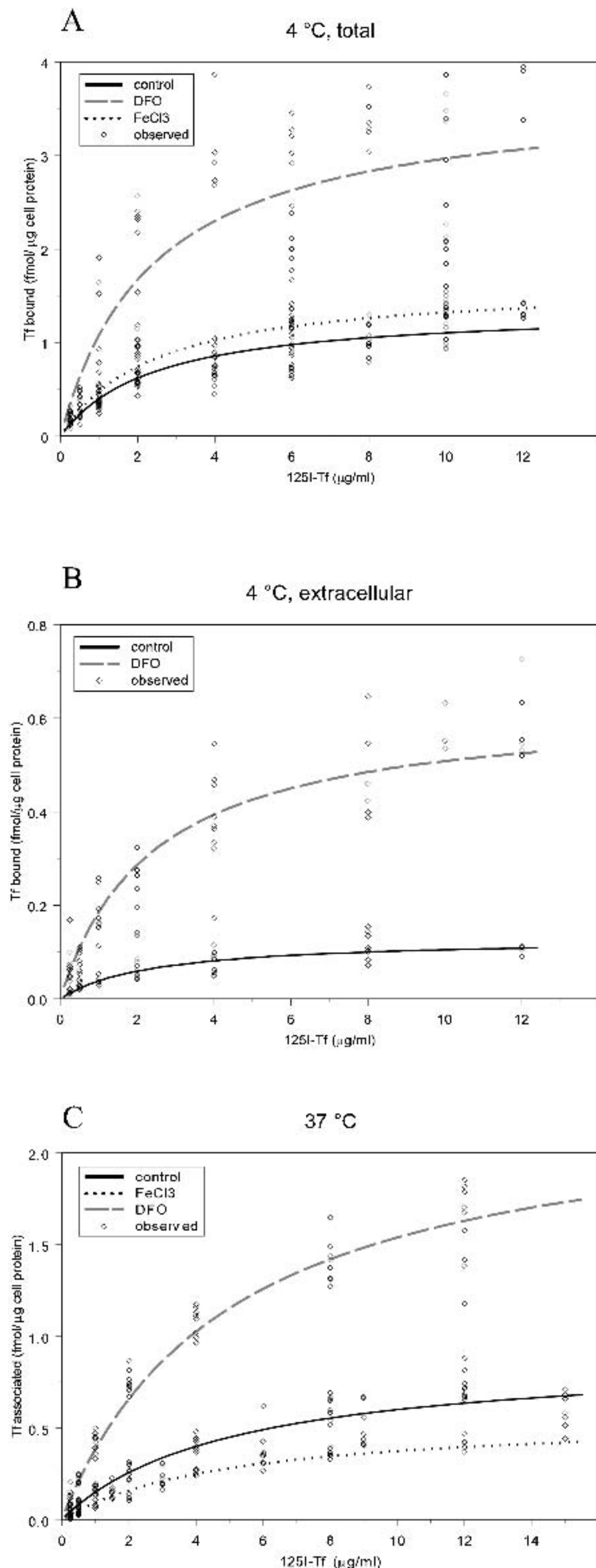

Fig. 1. (A) Total and (B) extracellular binding of ^{125}I-Tf to the TfR, determined at 4°C, and (C) association of ^{125}I-Tf by the TfR, determined at 37°C, on primary cultured BCECs in the presence of DFO or $FeCl_3$ (1 mM). Data represented are the curves of specific binding from at least three separate experiments performed in triplicate.

Table I. Total and Extracellular Expression, Determined at 4°C, of the TfR on Primary Cultured BCEC and the Association of Tf, Determined at 37°C in the Presence of DFO or $FeCl_3$ (1 mM)

	B_{max} (fmol/μg cell protein)		
	Total TfR expression	Extracellular TfR expression	Association
Control (CI)	1.37 (1.15–1.59)	0.13 (0.10–0.16)	0.90 (0.78–1.01)
DFO (CI)	3.68 (2.73–4.63)	0.63 (0.55–0.71)	2.31 (2.03–2.59)
$FeCl_3$ (CI)	1.64 (1.09–2.19)	Not detectable	0.56 (0.49–0.63)

Data were analyzed using NONMEM, K_d was estimated 2.4 ± 0.3 μg/ml and 5.0 ± 0.5 μg/ml for expression and association, respectively. Values for B_{max} are summarized in the table as mean (95% confidence interval, CI). Intra-individual residual variation was determined with a proportional error model and was 10% for expression levels and 9% for endocytosis.

Modulation by Astrocytic Factors or Tyrphostin A8

BCECS were cultured for 5 days in DMEM+ACM, DMEM+hep, or DMEM+S. After removal of endogenous Tf by preincubation with DMEM-S, BCECs were incubated with 8 μg/ml ^{125}I-Tf for 2 h at 4°C or for 1 h at 37°C. No significant differences in total or extracellular TfR expression level were obtained after culturing BCECs in DMEM+hep (0.04 ± 0.01 and 0.01 ± 0.004 ng Tf/μg cell protein, for total and extracellular TfR expression, respectively) or DMEM+S (0.05 ± 0.02 and 0.01 ± 0.002 ng Tf/μg cell protein, for total and extracellular TfR expression, respectively), compared to the control situation DMEM+ACM (0.04 ± 0.01 and 0.01 ± 0.005 ng Tf/μg cell protein, for total and extracellular TfR expression, respectively). The association experiments at 37°C showed a similar profile. After culturing BCECs in DMEM+hep or DMEM+S, association was 0.06 ± 0.03 or 0.07 ± 0.03 ng Tf/μg cell protein, respectively. After culturing BCECs in DMEM+ACM, association was 0.06 ± 0.02 ng Tf/μg cell protein.

To study the effect of T8, cells were preincubated with

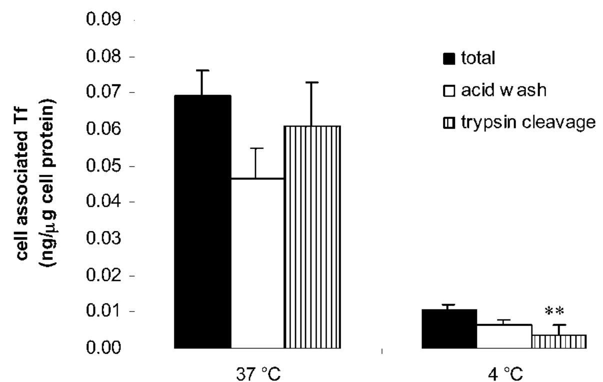

Fig. 2. Cell-associated ^{125}I-Tf at 37°C was reduced 20–30% after acid wash (citric acid, pH 5) or trypsin cleavage (0.25 mg/ml trypsin), indicating that 70–80% of the ^{125}I-Tf was endocytozed. At 4°C, the binding of ^{125}I-Tf is reduced to the level of nonspecific binding after trypsin cleavage, indicating that no radioligand was endocytosed. Data are represented as mean ± SD, one-way ANOVA shows no difference between groups at 37°C, but at 4°C there is a difference; $^{**}p < 0.01$ (trypsin cleavage vs. total; 4°C) Tukey-Kramer multiple comparison post-test.

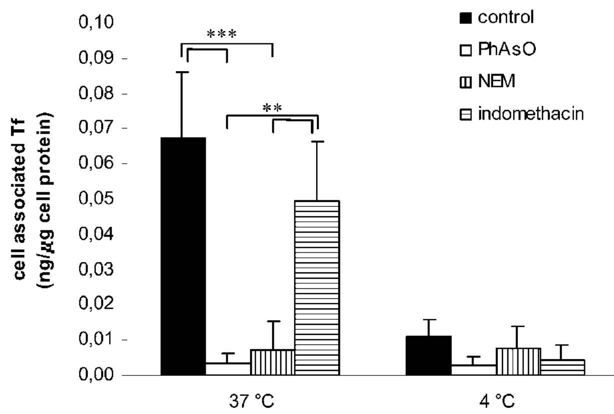

Fig. 3. Endocytosis, determined at 37°C, was inhibited after incubation with PhAsO (10 μM) and NEM (1 mM), but not after incubation with indomethacin (50 μg/ml), indicating that clathrin-mediated endocytosis is involved. At 4°C, none of these inhibitors had an effect on the extracellular binding of ^{125}I-Tf. Data represented are mean ± SD, one-way ANOVA shows a difference between groups at 37°C, but not at 4°C; ***$p < 0.001$ (PhAsO, NEM vs. control; 37°C), **$p < 0.01$ (PhAsO, NEM vs. indomethacin; 37°C), Tukey-Kramer multiple comparison post-test.

0.25 mM T8 for 10 min. This had no effect on the binding and association of ^{125}I-Tf. After 1 h incubation with the radioligand at 37°C, 0.045 ± 0.005 ng Tf/μg cell protein was associated, which was not different from the control (0.046 ± 0.012 ng Tf/μg cell protein). The extracellular binding was also not changed. Increasing the concentration T8 to 0.5 mM seems to down-regulate the association of Tf, as only 0.031 ± 0.006 ng Tf/μg cell protein was associated, though this is not statistically significant. Increasing the duration of the preincubation to 1 h did not change the effect of T8.

Inflammatory Disease Conditions

After preincubation of BCECs with LPS for 2 or 24 h, the total TfR expression level was decreased from 0.14 ± 0.03 ng Tf/μg cell protein to 0.07 ± 0.01 ng Tf/μg cell protein after 2 h ($p < 0.01$) and to 0.10 ± 0.03 ng Tf/μg cell protein after 24 h (not significant). For the extracellular TfR expression, as well as for the association experiments at 37°C, a similar effect was found (Figs. 4A and 4B). Preincubation of BCECs with 10 mM NAC for 1 h did not change the TfR expression level (0.10 ± 0.02 and 0.01 ± 0.002 ng Tf/μg cell protein for total and extracellular TfR, respectively) or association (0.05 ± 0.01 ng Tf/μg cell protein), but it did counteract the down-regulation of the TfR expression by a 2-h LPS preincubation when expressed in ng cell-associated Tf per μg cell protein (Figs. 4A and 4B). Increasing the preincubation time of NAC to 16 h (overnight) also had no effect on the TfR expression level and endocytosis, but it did prevent the down-regulation of the TfR after 2-h LPS preincubation. However, no changes in total and extracellular TfR expression levels and association were observed after preincubation with LPS when the data were corrected for cellular protein levels of the control situation Tf (Figs. 5A and 5B). Interestingly, the cellular protein levels were increased after 2-h preincubation with LPS (Table II), thereby decreasing the TfR expression level when expressed in ng cell-associated Tf per μg cell protein. Preincubation with NAC prevented the up-regulation of cellular

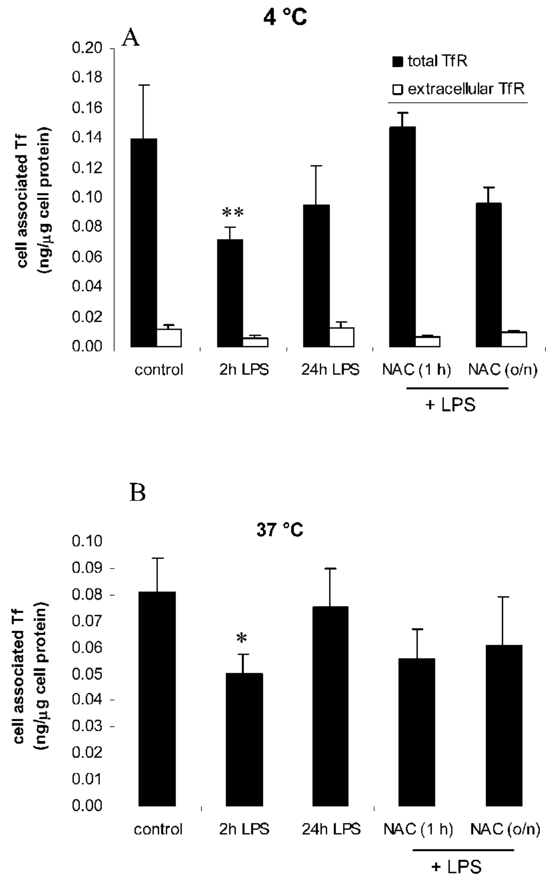

Fig. 4. (A) Total and extracellular binding of ^{125}I-Tf to the TfR, determined at 4°C, and (B) association of ^{125}I-Tf by the TfR, determined at 37°C, after preincubation with 100 ng/ml LPS for 2 or 24 h, or after combined preincubation with 10 mM NAC (1 h or overnight), followed by LPS preincubation (2 h, 100 ng/ml). Data represented are the specific binding corrected for the cellular protein levels from at least three separate experiments performed in triplicate. Statistical analysis was performed with one-way ANOVA (differences were found between groups at the total TfR level and at 37°C, but not at the extracellular TfR expression level); *$p < 0.05$, **$p < 0.01$ between groups, Tukey-Kramer multiple comparison post-test.

protein and, thereby, the apparent down-regulation of TfR when expressed in ng cell associated Tf per μg cell protein.

DISCUSSION

The passive transport of hydrophilic drugs to the CNS is limited by the BBB. This can be overcome by targeting drugs to transporters at the BBB. In addition, it has been shown that delivery of an exogenous gene, with liposomes targeted to the TfR on the BBB, exerts an effect in the brain *in vivo* (9). Therefore, this investigation focuses on the binding and internalization of Tf by the TfR, expressed on bovine BCECs, which are cultured in the presence of astrocytic factors. Furthermore, the influence of certain modulators and inflammatory disease conditions on the TfR expression and endocytosis was determined.

Binding studies at 4°C showed a B_{max} of 0.13 ± 0.02 fmol/μg protein and a K_d of 2.38 ± 0.32 μg/ml for BCECs cultured in the presence of astrocytic factors (Figs. 1A and 1B; Table I). This K_d corresponds to approximately 30 nM, which is consistent with the 10–75 nM range found in litera-

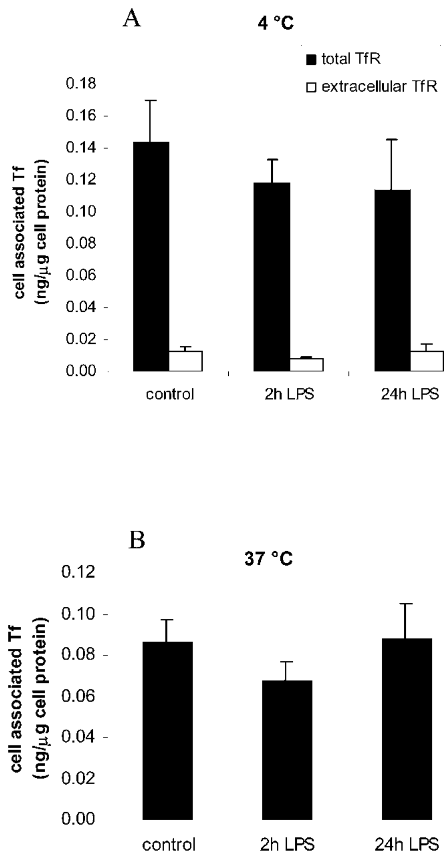

Fig. 5. (A) Total and extracellular binding of ^{125}I-Tf to the TfR, determined at 4°C, and (B) endocytosis of ^{125}I-Tf by the TfR, determined at 37°C, after preincubation with 100 ng/ml LPS (2 or 24 h) and expressed in ng cell-associated Tf corrected for cellular protein levels of the control situation. Data represented are the specific binding from at least three separate experiments performed in triplicate.

ture (22,23). It was possible to fully saturate the TfR after 2-h incubation with Tf concentrations up to 12 μg/ml. Binding experiments at 4°C were carried out for 2 h, as association and dissociation of ^{125}I-Tf had reached equilibrium at 2 h (data not shown). Permeabilizing the cells with saponin changed the observed B_{max} to 1.37 ± 0.11 fmol/μg protein (Table I). This shows that approximately 90% of the TfR is stored in an intracellular pool. This is consistent with observations by van Gelder *et al.* (27) and Raub and Newton (23), albeit that a higher total TfR expression level was observed in the latter studies. This might be due to differences in isolation and cell culture procedures, as they isolated BCECs directly and not brain capillaries from which BCECs were grown (13). Furthermore, in the latter investigations BCECs were not cultured in medium containing astrocytic factors. From literature (1), it is known that the endfeet of astrocytes play an important role in inducing and maintaining BBB characteristics, such as tight junctions between endothelial cells and expression of certain transporters. However, due to high non-specific binding of the iodinated Tf, it was not possible to perform binding studies in our *in vitro* BBB model, which is a co-culture of bovine BCEC and rat astrocytes (13). Therefore, in the current investigation, brain capillaries and BCECs were cultured in medium containing astrocytic factors.

Table II. Cellular Protein Levels After Pre-Incubation with 100 ng/ml LPS for 2 or 24 h, or After Combined Pre-Incubation with 10 mM NAC (1 h or Overnight) and 100 ng/ml LPS (2 h)

	Cellular protein levels (μg/well)		
	4°C	4°C + Saponin	37°C
Control	22.9 ± 5.3	7.2 ± 3.0	21.3 ± 4.6
LPS (2 h)	28.2 ± 3.7	11.9* ± 2.3	31.6* ± 4.9
LPS (24 h)	23.4 ± 2.1	8.6 ± 2.9	24.8 ± 7.0
NAC (1 h) + LPS (2 h)	25.3 ± 1.9	9.0 ± 0.8	26.0 ± 4.0
NAC (on) + LPS (2 h)	24.5 ± 1.4	6.0 ± 1.5	24.9 ± 3.3

* $P < 0.05$, LPS (2 h) vs control (4°C + saponin and 37°C) and LPS (2 h) vs NAC (on) + LPS (4°C + saponin), ANOVA analysis with Tukey-Kramer multiple comparisons post-test. Data are mean ± s.d. from at least 3 experiments in triplicate.

By using a population approach, it was possible to fit all saturation experiments simultaneously, thereby obtaining a single unique estimate of the affinity constant K_d and separate B_{max} values for all situations. In order to study the modulation of the TfR by iron, we preincubated BCECs with either $FeCl_3$ or with DFO. DFO is an iron scavenger, which appears to distribute into both intra- and extracellular spaces (19). Incubation with DFO resulted into an increase in B_{max} for both the total (3.68 ± 0.48 fmol/μg cell protein) as well as the extracellular (0.63 ± 0.04 fmol/μg cell protein) TfR expression level (Figs. 1A and 1B; Table I). Interestingly, the extracellular TfR expression is increased 4.8-fold, whereas the total TfR expression is only increased 2.7-fold. Thus, the extracellular increase is not only due to synthesis of new receptors, but also to a shift toward the outside of the BCECs. These experiments show that DFO increases the expression of the TfR, which could be useful for improved drug targeting to this receptor. DFO (desferal) is currently used for treatment of (transfusional) iron overload, but it has many side effects (28). *In vivo* up-regulation of the TfR may still be possible when new iron chelators are developed.

Addition of an excess of iron did not change the total TfR expression level, but at the extracellular TfR level it was not possible to detect a specific signal. This might be due to the disappearance of the TfR from the cell surface.

Experiments, performed at 37°C, showed a B_{max} of 0.90 ± 0.06 fmol/μg protein, which is the maximal receptor occupancy as a combination of binding and endocytosis, and a K_d of 5.03 ± 0.50 μg/ml (Fig. 1C; Table I). Incubation time was set to 1 h, as equilibrium between association and dissociation of ^{125}I-Tf was reached (data not shown). Preincubation with DFO resulted in a 2.6-fold increase of B_{max} at 37°C, whereas preincubation with $FeCl_3$ resulted in a 1.6-fold decrease (Fig. 1C, Table I). In these full saturation experiments, we have not discriminated between internalized and extracellularly bound ^{125}I-Tf, nor have we investigated the retro-endocytosis of apo-Tf. Zhang and Pardridge (29) have shown that apo-Tf is rapidly effluxed from the brain. However, this does not necessarily suggest that the TfR is not suitable for drug targeting, as it is not investigated what happens with a Tf-drug conjugate or with Tf-tagged liposomes. In addition, it has been demonstrated that drug and gene transfer to the brain was successful following targeting to the TfR (9). The changes in B_{max} at 37°C are consistent with the change that was found in extracellular TfR expression levels at 4°C. Increasing the con-

centrations DFO and iron to 10 mM did not have an additional effect on the binding or endocytosis of ^{125}I-Tf.

In the analysis of these full saturation data, we initially estimated separate values for K_d. For experiments performed at 4°C, K_d values for extracellular binding were 3.2 ± 1.0 μg/ml and 4.5 ± 1.0 μg/ml for control and DFO, respectively, whereas for the total binding, K_d values of 2.2 ± 0.5, 3.2 ± 0.9, and 3.0 ± 0.5 μg/ml were obtained for control, DFO, and $FeCl_3$, respectively. Association experiments at 37°C showed K_d values of 5.2 ± 0.9, 4.2 ± 0.8, and 5.5 ± 0.7 μg/ml for control, DFO, and FeCl3, respectively. Statistical analysis has shown no difference between the K_d values for the binding or between the K_d values for the association. Therefore, a population approach was used, yielding a greater statistical power to determine the differences between up- and down-regulation of the TfR. Incorporation of interoccasion variability in the modeling was considered. However, this did not contribute to the goodness of fit, as the triplicate measurements showed a very small interoccasion variability. Several features of the stochastic model were investigated, which included interindividual errors on all or some parameters. Furthermore, in the structural model, the incorporation of a Hill factor was investigated. Based on the individual and population predictions, fitted through the data points, the intraindividual residual variation, and the minimal value of the objective function, we concluded that addition of interindividual variability on one or more parameters did not improve the goodness-of-fit. Incorporation of a Hill slope did not improve the fit either. Because it is known from literature that the binding of Tf to its receptor is 1:1, it is indeed justified to assume that the Hill slope is 1.

To study further the extent and mechanism of endocytosis of Tf by acid wash, trypsin cleavage, or with inhibitors, a concentration of 8 μg/ml ^{125}I-Tf was used, as full saturation of the TfR is reached at this concentration. After acid wash or trypsin cleavage, 70–80% of the added ^{125}I-Tf remained cell-associated at 37°C (Fig. 2). This was not significantly different from the total association, indicating that the TfR is actively endocytosing most of its ligand. At 4°C, trypsin cleavage removed ^{125}I-Tf to the level of nonspecific binding, whereas with acid wash approximately 50% of the added Tf was removed. This rather inefficient removal might be due to the relatively high pH (pH 5.0) used for acid wash (24). Mostly, acid wash (28,29) is performed at pH 3, but under those circumstances most of the BCECs are damaged in our experiments.

To study the process, by which the TfR is endocytosing Tf, several inhibitors were used, as is shown in Fig. 3. PhAsO and NEM inhibited the endocytosis of ^{125}I-Tf almost completely, whereas indomethacin had no significant effect. None of these inhibitors had an effect on the binding of ^{125}I-Tf, indicating that they did not alter the binding properties of Tf to the TfR. PhAsO is known to inhibit clathrin-mediated internalization, which is associated with receptor-mediated endocytosis (14,30). In this respect it was used to examine the contribution of receptor mediated endocytosis to the total intracellular uptake of Tf (31). NEM is a nonspecific inhibitor of endocytotic processes, as it binds to a NEM-sensitive factor, which is responsible for the budding and fusion of a wide range of vesicles (15). Indomethacin reduces the number of plasmalemmal caveolae (15), thereby inhibiting caveolae-mediated endocytosis. Because PhAsO and NEM reduced the endocytosis of ^{125}I-Tf almost completely, whereas indomethacin had no effect, we conclude that TfR-ligand endocytosis is mediated via clathrin-coated vesicles.

Modulation of the TfR was investigated not only by scavenging or extra addition of iron, but also by other modulators such as astrocytic factors. However, due to high nonspecific binding, it was not possible to perform experiments in a coculture of BCECs and astrocytes (13), in which the BCECs are in direct contact with the astrocytes. Therefore, all experiments were performed in DMEM+ACM. This is important because it is known from literature that astrocytic factors induce and maintain BBB properties of the BCECs (1). In our experiments, removing astrocytic factors from the cell culture medium did not change the total TfR expression level, as this was 0.04 ± 0.01 and 0.05 ± 0.02 ng Tf/μg cell protein for DMEM+ACM and DMEM+S, respectively. Also for the extracellular TfR expression level and the association, no differences were found in the presence or absence of ACM. The iron and Tf content in ACM were 3.3 and 7.3 μM, respectively, which is not different from 3.5 μM Fe and 5.2 μM Tf found for DMEM+S. Therefore, it can be concluded that astrocytes had not secreted extra iron or Tf. Furthermore, there appear to be no other astrocytic factors secreted in ACM, which influence the TfR expression and endocytosis.

To increase endocytosis of Tf by BCECs, we also investigated the effect of the GTP-ase inhibitor T8, as it was shown in literature that T8 enhanced the transcytosis of Tf-conjugates through Caco-2 cells (20). Ten-minute preincubation with 0.25 mM T8 did not change Tf binding nor association. Increasing the concentration to 0.5 mM revealed the tendency to decrease the association of Tf. Therefore, we conclude from our experiments that T8 does not enhance endocytosis (as a first step of transcytosis) of Tf in BCECs. The difference between literature data (20) and our results may be explained by differences in cell type (endothelium vs. epithelium), cellular metabolism (34), and species (bovine vs human).

The modulators that we have applied in the investigations described so far are either involved in the basic modulation of the TfR (iron), in the functional modulation of the BCECs (ACM), or the interference with intracellular processes (T8) and were tested at physiological conditions. In addition, we were also interested in the expression of the TfR under disease conditions, to investigate the possibility to target drugs to the BBB or brain under these conditions. Therefore, we have applied LPS, an inducer of inflammatory disease conditions, and the free-radical scavenger NAC, as an inhibitor of inflammatory disease conditions. The effects of LPS on the binding and association after 24-h preincubation were small (Figs. 4 and 5). Following a 2-h preincubation, the expression of the TfR was not changed (data not shown). However, due to the increase in the concentration of cellular protein (Table II), the TfR expression per μg of cellular protein was decreased (Fig. 4A). Because shortly after inflammation, so-called acute phase proteins are formed (35), which can cause increased cellular protein levels, we have evaluated the effect of LPS on TfR expression, without correcting for this increase (Fig. 5). Therefore, we have corrected the TfR expression for the cellular protein of the control situation and not for the 2 h or 24 h LPS situation. In addition, the mRNA level of the TfR was also not changed after 2-h preincubation with LPS (personal communication with Dr. P. J. Gaillard),

confirming that LPS has no effect on TfR expression level. Our results are consistent with the findings of Hallmann *et al.* (31), who have studied the TfR in a murine brain derived endothelial cell line, cultured without astrocytic factors, in which they did not find an effect of LPS on Tf endocytosis. Experiments conducted with cell lines of non-brain origin showed a decrease in TfR mRNA (36) or expression levels (21) due to LPS or oxidative stress. Our results indicate that the TfR at the BBB is expressed equally under physiological conditions as well as inflammatory disease conditions.

The free-radical scavenger NAC prevented disturbances of the BBB by LPS (26) and was also able to prevent down-regulation of the TfR caused by oxidative stress in K562 cells (21). Therefore, we have investigated the effect of NAC on the expression of the TfR on BCEC. One hour or overnight preincubation with 10 mM NAC did not affect the TfR expression or association, nor did it affect the cellular protein levels (data not shown). In addition, the combination of NAC (1 h or overnight) and LPS (2 h) did not have an effect on the TfR expression levels nor endocytosis (Fig. 4). However, NAC was able to counteract the up-regulation of cellular protein levels caused by LPS (Table II), resulting in "no change" in TfR expression per μg cellular protein compared to control.

In conclusion, we have characterized the TfR on BCECs cultured in the presence of astrocytic factors. Although this receptor is present at the BBB, it is also present at other tissues. Our current research on primary cultured BCECs gives a extensive overview of the capacity and the use of the TfR at the BBB for drug delivery. The extracellular expression level of TfR is only 10% of the total TfR expression, but the association of Tf is high. Furthermore, it was shown that 70–80% of Tf is endocytosed by the TfR via clathrin-coated vesicles within 1 h. With DFO it was possible to increase the TfR expression level, as well as the association. This could have important implications for drug targeting to the brain via the TfR. In all other experimental conditions, including high iron concentrations, removal of ACM, inhibition of GTP-ase, and inflammatory conditions, the TfR expression level, as well as the association, was not changed. This shows that the TfR is present on the BBB in physiological as well as inflammatory disease conditions. Due to its constant expression, the TfR is an interesting target for the selective delivery of (hydrophilic) drugs to the brain.

ACKNOWLEDGMENTS

The authors thank Dr. Pieter Gaillard for his helpful discussion and technical comments, Dymphy Huntjens (M.Sc.) for her input toward the NONMEM data analysis, and Dr. Marijke Frohlich from the Central Clinical Laboratorium for iron and Tf measurements in ACM. This work was financially supported by STIGO (stimulation for innovative drug research, ZonMw) grant 014-80-003, The Hague, The Netherlands.

REFERENCES

1. L. L. Rubin and J. M. Staddon. The cell biology of the blood-brain barrier. *Annu. Rev. Neurosci.* **22**:11–28 (1999).
2. W. M. Pardridge. Blood-brain barrier biology and methodology. *J. Neurovirol.* **5**:556–569 (1999).
3. W. M. Pardridge. Drug delivery to the brain. *J. Cereb. Blood Flow Metab.* **17**:713–731 (1997).
4. A. G. de Boer, I. C. van der Sandt, and P. J. Gaillard. The role of drug transporters at the blood-brain barrier. *Annu. Rev. Pharmacol. Toxicol.* **43**:629–656 (2003).
5. W. Pardridge. Drug targeting, drug discovery, and brain drug development, *Brain Drug Targeting: The Future of Brain Drug Development*, Press Syndicate of the University of Cambridge, Cambridge, 2001, pp. 1–12.
6. K. R. Duffy and W. M. Pardridge. Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res.* **420**:32–38 (1987).
7. B. Dehouck, L. Fenart, M. P. Dehouck, A. Pierce, G. Torpier, and R. Cecchelli. A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J. Cell Biol.* **138**: 877–889 (1997).
8. H. E. de Vries, B. Breedveld, J. Kuiper, A. G. de Boer, T. J. Van Berkel, and D. D. Breimer. High-density lipoprotein and cerebral endothelial cells in vitro: interactions and transport. *Biochem. Pharmacol.* **50**:271–273 (1995).
9. N. Shi, Y. Zhang, C. Zhu, R. J. Boado, and W. M. Pardridge. Brain-specific expression of an exogenous gene after i.v. administration. *Proc. Natl. Acad. Sci. USA* **98**:12754–12759 (2001).
10. T. Moos and E. H. Morgan. Transferrin and transferrin receptor function in brain barrier systems. *Cell. Mol. Neurobiol.* **20**:77–95 (2000).
11. E. Baker and E. H. Morgan. Iron Transport. In J. H. Brock, J. W. Halliday, M. Pippard, and L. W. Powell (eds.), *Iron Metabolism in Health and Disease*, Saunders, London, 1994, pp. 63–95.
12. D. Trinder and E. Baker. Transferrin receptor 2: a new molecule in iron metabolism. *Int. J. Biochem. Cell Biol.* **35**:292–296 (2003).
13. P. J. Gaillard, L. H. Voorwinden, J. L. Nielsen, A. Ivanov, R. Atsumi, H. Engman, C. Ringbom, A. G. de Boer, and D. D. Breimer. Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur. J. Pharm. Sci.* **12**:215–222 (2001).
14. H. M. Nguyen, C. M. Cahill, P. S. McPherson, and A. Beaudet. Receptor-mediated internalization of [3H]-neurotensin in synaptosomal preparations from rat neostriatum. *Neuropharm.* **42**: 1089–1098 (2002).
15. M. Gumbleton, A. G. Abulrob, and L. Campbell. Caveolae: an alternative membrane transport compartment. *Pharm. Res.* **17**: 1035–1048 (2000).
16. J. L. Casey, D. M. Koeller, V. C. Ramin, R. D. Klausner, and J. B. Harford. Iron regulation of transferrin receptor mRNA levels requires iron- responsive elements and a rapid turnover determinant in the 3′ untranslated region of the mRNA. *EMBO J.* **8**:3693–3699 (1989).
17. L. C. Kuhn. mRNA-protein interactions regulate critical pathways in cellular iron metabolism. *Br. J. Haematol.* **79**:1–5 (1991).
18. W. van Gelder, M. I. Huijskes-Heins, M. I. Cleton-Soeteman, J. P. van Dijk, and H. G. van Eijk. Iron uptake in blood-brain barrier endothelial cells cultured in iron- depleted and iron-enriched media. *J. Neurochem.* **71**:1134–1140 (1998).
19. K. R. Bridges and A. Cudkowicz. Effect of iron chelators on the transferrin receptor in K562 cells. *J. Biol. Chem.* **259**:12970–12977 (1984).
20. C. Q. Xia and W. C. Shen. Tyrphostin-8 enhances transferrin receptor-mediated transcytosis in Caco-2- cells and inreases hypoglycemic effect of orally administered insulin-transferrin conjugate in diabetic rats. *Pharm. Res.* **18**:191–195 (2001).
21. W. Malorni, U. Testa, G. Rainaldi, E. Tritarelli, and C. Peschle. Oxidative stress leads to a rapid alteration of transferrin receptor intravesicular trafficking. *Exp. Cell Res.* **241**:102–116 (1998).
22. L. Descamps, M. P. Dehouck, G. Torpier, and R. Cecchelli. Receptor-mediated transcytosis of transferrin through blood-brain barrier endothelial cells. *Am. J. Physiol.* **270**:H1149–H1158 (1996).
23. T. J. Raub and C. R. Newton. Recycling kinetics and transcytosis of transferrin in primary cultures of bovine brain microvessel endothelial cells. *J. Cell. Physiol.* **149**:141–151 (1991).
24. J. A. Koenig, J. M. Edwardson, and P. P. Humphrey. Somatostatin receptors in Neuro2A neuroblastoma cells: ligand internalization. *Br. J. Pharmacol.* **120**:52–59 (1997).
25. E. A. Rutledge, C. A. Mikoryak, and R. K. Draper. Turnover of

the transferrin receptor is not influenced by removing most of the extracellular domain. *J. Biol. Chem.* **266**:21125–21130 (1991).
26. P. J. Gaillard, A. G. de Boer, and D. D. Breimer. Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro. *Microvasc. Res.* **65**: 24–31 (2003).
27. W. van Gelder, M. I. Huijskes-Heins, J. P. van Dijk, M. I. Cleton-Soeteman, and H. G. van Eijk. Quantification of different transferrin receptor pools in primary cultures of porcine blood-brain barrier endothelial cells. *J. Neurochem.* **64**:2708–2715 (1995).
28. R. S. Britton, K. L. Leicester, and B. R. Bacon. Iron toxicity and chelation therapy. *Int. J. Hematol.* **76**:219–228 (2002).
29. Y. Zhang and W. M. Pardridge. Rapid efflux from brain to blood across the blood-brain barrier. *J. Neurochem.* **76**:1597–1600 (2001).
30. M. B. Wheeler, R. W. Gelling, S. A. Hinke, B. Tu, R. A. Pederson, F. Lynn, J. Ehses, and C. H. McIntosh. Characterization of the carboxyl-terminal domain of the rat glucose- dependent insulinotropic polypeptide (GIP) receptor. A role for serines 426 and 427 in regulating the rate of internalization. *J. Biol. Chem.* **274**:24593–24601 (1999).
31. W. M. Pardridge and J. Eisenberg. and J. Yang. Human blood-brain barrier transferrin receptor. *Metabolism* **36**:892–895 (1987).
32. M. Wilcke and E. Walum. Characterization of leptin intracellular trafficking. *Eur. J. Histochem.* **44**:325–334 (2000).
33. R. Hallmann, D. L. Savigni, E. H. Morgan, and E. Baker. Characterization of iron uptake from transferrin by murine endothelial cells. *Endothelium* **7**:135–147 (2000).
34. A. G. de Boer, P. J. Gaillard, and D. D. Breimer. The transference of results between blood-brain barrier cell culture systems. *Eur. J. Pharm. Sci.* **8**:1–4 (1999).
35. H. Baumann. and J. Gauldie. The acute phase response. *Immunol. Today* **15**:74–80 (1994).
36. S. Kim and P. Ponka. Effects of interferon-gamma and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. *J. Biol. Chem.* **275**:6220–6226 (2000).