Epinephrine enhances lysosomal enzyme delivery across the blood–brain barrier by up-regulation of the mannose 6-phosphate receptor

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Contributed by William S. Sly, June 18, 2007 (sent for review May 16, 2007)

Delivering therapeutic levels of lysosomal enzymes across the blood–brain barrier (BBB) has been a pivotal issue in treating CNS storage diseases, including the mucopolysaccharidoses. An inherited deficiency of β-glucuronidase (GUS) causes mucopolysaccharidosis type VII that is characterized by increased systemic and CNS storage of glycosaminoglycans. We previously showed that the neonate uses the mannose 6-phosphate (M6P) receptor to transport phosphorylated GUS (P-GUS) across the BBB and that this transporter is lost with maturation. Induction of expression of this BBB transporter would make enzyme replacement therapy in the adult possible. Here, we tested pharmacological manipulation with epinephrine to restore functional transport of P-GUS across the adult BBB. Epinephrine (40 nmol) coinjected i.v. with ^{131}I-P-GUS induced the transport across the BBB in 8-week-old mice. The brain influx rate of ^{131}I-P-GUS (0.29 μl/g per min) returned to the level seen in neonates. Capillary depletion showed that 49% of the ^{131}I-P-GUS in brain was in brain parenchyma. No increases of influx rate or the vascular space for ^{125}I-albumin, a vascular marker, was observed with epinephrine (40 nmol), showing that enhanced passage was not caused by disruption of the BBB. Brain uptake of ^{131}I-P-GUS was significantly inhibited by M6P in a dose-dependent manner, whereas epinephrine failed to increase brain uptake of nonphosphorylated GUS. Thus, the effect of epinephrine on the transport of ^{131}I-P-GUS was ligand specific. These results indicate that epinephrine restores the M6P receptor-mediated functional transport of ^{131}I-P-GUS across the BBB in adults to levels seen in the neonate.

β-glucuronidase | drug delivery | enzyme replacement therapy | lysosomal storage disease | phosphorylated β-glucuronidase

Mucopolysaccharidosis type VII (MPS VII) is characterized by abnormal lysosomal storage of glycosaminoglycans (GAGs) in most tissues including the CNS. MPS VII is caused by an inherited deficiency of β-glucuronidase (β-D-glucuronoside glucuronosohydrolase; EC 3.2.1.31) (GUS), an enzyme that cleaves glucuronic acid residues from nonreducing termini of GAGs. Enzyme replacement therapy (ERT) with murine or human GUS in MPS VII mice reduces visceral lysosomal storage, normalizes the pathological phenotype, and prolongs lifespan (1–3). It also improves abnormal storage in brain if treatment with GUS is begun before 2 weeks of age (2, 4). Delivering the enzyme to treat CNS storage disease has been a key problem because the blood–brain barrier (BBB) restricts the passage into brain of exogenously administrated enzymes. In a prior study, we found that human phosphorylated GUS (P-GUS) is transported across the neonatal BBB by mannose 6-phosphate (M6P) receptor-mediated transcytosis, and that this transport mechanism is progressively lost with maturation so that, by 7 weeks, mice have little or no transport left (5). Thus, alterations in the transport of a lysosomal enzyme across the BBB occur with postnatal development, which limits the effectiveness of ERT to adults. Sly *et al.* (6) and Stahl *et al.* (7) have demonstrated that the terminal half-life of P-GUS is ≈5 min after i.v. injection in MPS VII mice and plasma clearance of P-GUS is predominantly mediated by the mannose receptor *in vivo*. Saturating the mannose receptor with a high dose of enzyme may improve targeting to M6P receptor-containing tissues. In fact, high-dose ERT in adult MPS VII mice was partially successful in clearing CNS storage (8). Partial correction of lysosomal storage in brain by high-dose ERT has also been reported in three other murine models of lysosomal storage disease [aspartylglycosaminuria (9), α-mannosidosis (10), and metachromatic leukodystrophy (11)]. However, ERT to treat CNS storage is still challenging because the BBB restricts the entry of the enzyme to the majority of brain cells.

Several studies have shown that BBB transporters can be modified by pharmacological manipulations. Transport of leptin across the BBB is transiently enhanced by α-adrenergic agents *in vivo* without affecting the integrity of the BBB (12). It has also been reported that α- and β-adrenoceptors mediate changes in the permeability in bovine brain microvessel endothelial cells, an *in vitro* model of the BBB. Both α- and β-adrenoreceptor-mediated changes in permeability are abolished by inhibiting fluid-phase pinocytosis (13, 14).

If the transport mechanism across the BBB for a lysosomal enzyme, which is operative in neonates, could be restored, lysosomal enzymes containing the M6P moiety might be delivered into the brain at therapeutically effective levels, in which case CNS storage diseases could be treated. Here, we report the restoration of M6P receptor-mediated transport of the lysosomal enzyme GUS at the adult BBB by pharmacological manipulation with epinephrine.

Results

Brain Influx of ^{131}I-P-GUS from Serum After i.v. Injection. The concentration-time profile of ^{131}I-P-GUS and ^{125}I-albumin in the serum fraction up to 10 min after the i.v. injection is shown in Fig. 1. The serum concentration of ^{131}I-P-GUS declined with time after i.v. injection in 8-week-old mice, as expected from previously demonstrated mannose- and M6P receptor-mediated clearance systems (15, 16). In contrast, the levels of ^{125}I-albumin in serum were sustained throughout the experiment. Epinephrine at the doses of 12, 40, and 120 nmol per mouse slightly increased the serum levels of ^{131}I-P-GUS, and the levels of ^{125}I-albumin remained unchanged, regardless of the dose.

Author contributions: A.U., W.A.B., and W.S.S. designed research; A.U. and J.H.G. performed research; A.U. and J.H.G. contributed new reagents/analytic tools; A.U., J.H.G., W.A.B., and W.S.S. analyzed data; and A.U., W.A.B., and W.S.S. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: BBB, blood–brain barrier; ERT, enzyme replacement therapy; GUS, β-glucuronidase; P-GUS, phosphorylated GUS; NP-GUS, nonphosphorylated GUS; ID, injected dose; MPS VII, mucopolysaccharidosis type VII; M6P, mannose 6-phosphate.

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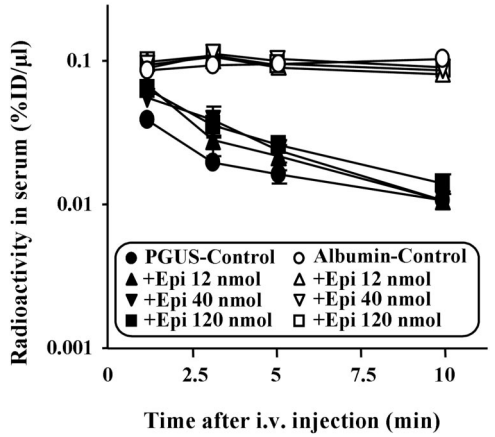

Fig. 1. Time courses of radioactivity in serum after i.v. coinjection of ^{131}I-P-GUS and ^{125}I-albumin in 8-week-old mice. Mice received ^{131}I-P-GUS and ^{125}I-albumin (5.5×10^5 cpm of each) i.v. and were then killed 1, 3, 5, or 10 min after the injection. Epinephrine (Epi; 0, 12, 40, or 120 nmol) was included in the injection solution. Each point represents the mean ± SE of three to six mice.

The effects of epinephrine on the entry of ^{131}I-P-GUS and ^{125}I-albumin into brain were also examined (Fig. 2). The coinjection of epinephrine produced a rapid increase in the ^{131}I-P-GUS taken up by the brain, as calculated from Eq. **3**, in a dose-dependent manner. The maximal effects were observed 5 min after the injection. In contrast, in the absence of coinjected epinephrine, the control group showed negligible uptake of ^{131}I-P-GUS into the brain during the course of the experiment. Thus, adult mice show an epinephrine-induced uptake of P-GUS in the brain, even though the rate of clearance from serum was not detectably affected by epinephrine. At the highest dose of epinephrine, the uptake of ^{125}I-albumin also showed a significant increase 5 min after the injection, although a smaller increase than that seen for P-GUS.

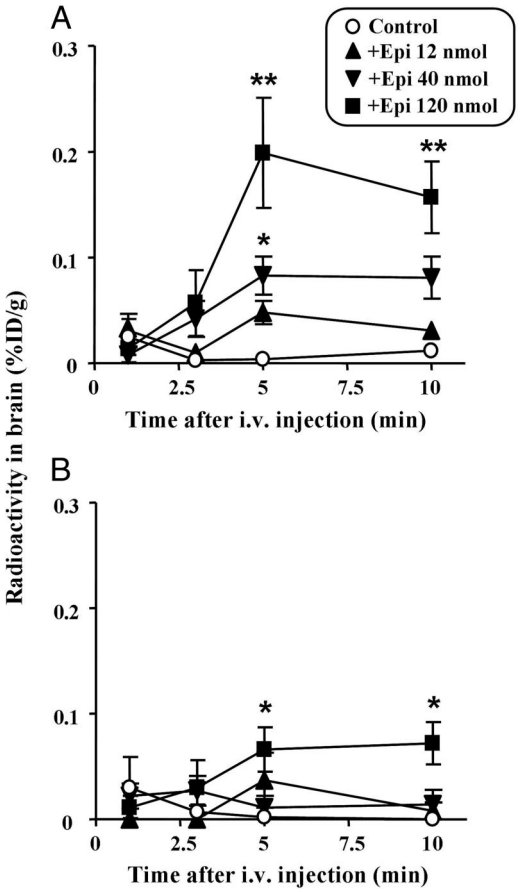

Fig. 2. The percent of ^{131}I-P-GUS (*A*) and ^{125}I-albumin (*B*) taken up by the brain after i.v. injection in 8-week-old mice. ^{131}I-P-GUS, ^{125}I-albumin (5.5×10^5 cpm of each), and epinephrine (Epi; 12, 40, and 120 nmol) were coinjected. Asterisks indicate a significant difference in comparison to the control at each time point: *, $P < 0.05$ and **, $P < 0.01$. Each point represents the mean ± SE of three to six mice.

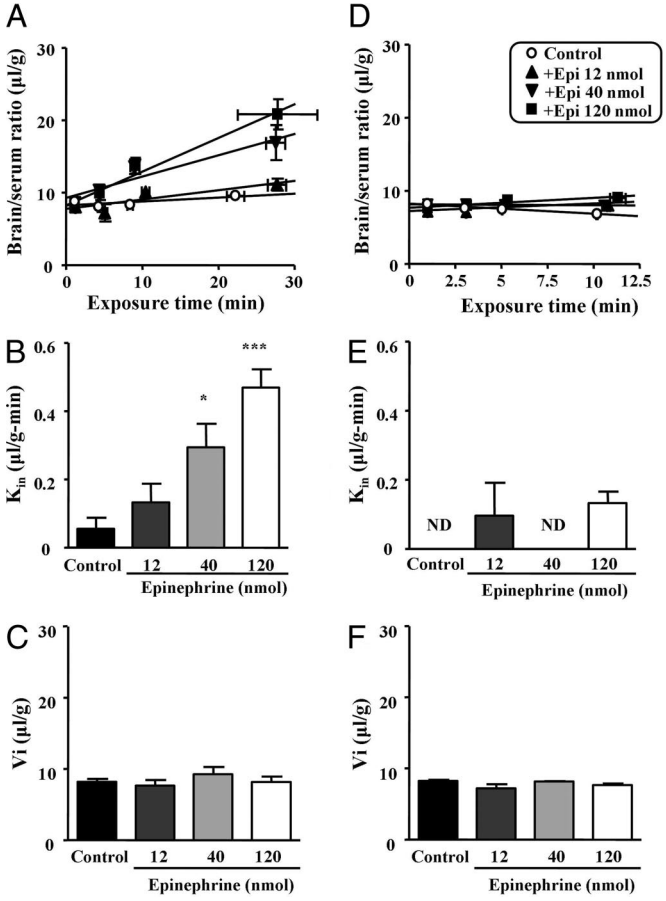

Fig. 3. The multiple-time regression analysis of brain-to-serum ratios of ^{131}I-P-GUS (*A–C*) and ^{125}I-albumin (*D–F*). (*A* and *D*) The relation between the brain-to-serum ratio and the exposure time of ^{131}I-P-GUS (*A*) and ^{125}I-albumin (*D*) is shown. (*B* and *E*) The influx rates (K_{in}) as determined by multiple-time regression analysis of ^{131}I-P-GUS (*B*) and ^{125}I-albumin (*E*) are shown. Asterisks indicate significant differences in comparison to the control: *, $P < 0.05$; ***, $P < 0.001$. ND, not detected (no blood-to-brain entry). (*C* and *F*) The initial volumes (V_i) of distribution of ^{131}I-P-GUS (*C*) and ^{125}I-albumin (*F*) are presented. Each point represents the mean ± SE of three to six mice.

Blood-to-Brain Unidirectional Influx Rate (K_{in}). Fig. 3 *A* and *D* shows the multiple-time regression analysis of brain/serum ratios for ^{131}I-P-GUS and ^{125}I-albumin, respectively. The epinephrine-induced increase in the transport seen for ^{131}I-P-GUS was not seen for the coinjected ^{125}I-albumin, indicating the effect was ligand specific. Whereas the slopes of ^{131}I-P-GUS significantly differed at the various doses from each other [$F(3,8) = 12.623$; $P = 0.00211$], the slope in the control group did not significantly deviate from zero [$F(1,2) = 2.958$; $P = 0.2276$], suggesting there is no significant influx of P-GUS without epinephrine. Fig. 3 *B* and *E* compares the dose–response for epinephrine on the influx

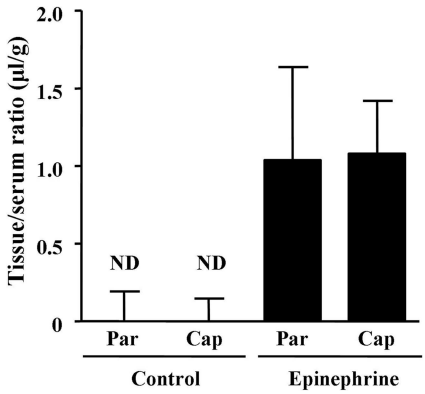

Fig. 4. Distribution volumes of ^{131}I-P-GUS in brain parenchyma (Par) and capillary (Cap) fraction 10 min after i.v. coinjection of epinephrine (40 nmol) in 8-week-old mice. Vascular spaces as simultaneously measured by ^{125}I-albumin were subtracted from each fraction. Each column represents the mean ± SE of four mice. ND, not detected.

rate of ^{131}I-P-GUS and ^{125}I-albumin, respectively. ^{131}I-P-GUS, but not ^{125}I-albumin, influx was significantly enhanced by 40 and 120 nmol of epinephrine per mouse. The influx rates of ^{131}I-P-GUS in control and epinephrine (12, 40, and 120 nmol)-treated groups were 0.06 ± 0.03 (control), 0.13 ± 0.05 (12 nmol), 0.29 ± 0.07 (40 nmol; $P < 0.05$), and 0.47 ± 0.05 (120 nmol; $P < 0.001$) μl/g per min, respectively. Fig. 3 *C* and *F* shows the initial volumes of distribution for ^{131}I-P-GUS and ^{125}I-albumin, which were unchanged by epinephrine. From these results, the epinephrine-induced transport into adult brain is shown to be ligand-specific and not related to vascular leakage, BBB disruption, or expansion of the brain's vascular space.

Evidence that P-GUS Is Transported into Brain Parenchyma. To determine the extent to which the brain capillaries comprising the BBB retained ^{131}I-P-GUS rather than transporting it completely across the capillary wall and into brain parenchyma, a capillary depletion study was conducted 10 min after i.v. coinjection of ^{131}I-P-GUS and ^{125}I-albumin with or without injection of epinephrine at the dose of 40 nmol per mouse (Fig. 4). In the absence of coinjected epinephrine, the tissue/serum ratio of ^{131}I-P-GUS was below the level of detection in both the parenchyma and capillary fractions when compared with the distribution of ^{125}I-albumin. With coinjection of epinephrine, ^{131}I-P-GUS was detectable in both the parenchymal fraction (1.04 ± 0.60 μl/g) and the capillary fraction (1.08 ± 0.34 μl/g). Thus, 49.1% of the ^{131}I-P-GUS taken up by brain had actually entered the brain parenchyma by 10 min after the treatment with epinephrine, and 50.9% remained in the capillary fraction 10 min after the injection. These results suggest that epinephrine induces transcytosis across the BBB in the adult as has been demonstrated in neonates without epinephrine treatment (5).

In earlier work, we demonstrated by HPLC that P-GUS found in the parenchymal fraction of brain after infusing the neonate was intact enzyme with 87% of the radioactivity still in a high molecular weight form. To confirm transport of the intact P-GUS in adults in the present study, we infused 10 mg/kg of nonradioactive P-GUS into adult, GUS-deficient MPS VII mice with or without coinjected epinephrine (40 nmol) and measured the activity in whole brain from perfused animals 24 h after injection. Control, noninjected mice had 0.1 ± 0.03 units/ng of activity. Those infused with P-GUS without epinephrine had 1.15 ± 0.36 units/ng activity. Those receiving P-GUS + 40 nmol epinephrine had 2.09 ± 0.34 units/ng activity. Epinephrine enhanced uptake of P-GUS activity into the brain by 78%, in this

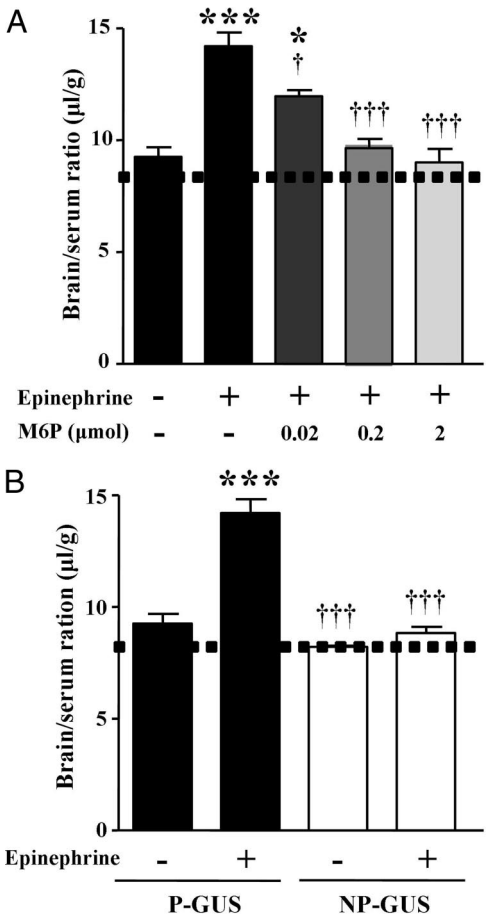

Fig. 5. M6P dependence of epinephrine-induced brain uptake of GUS in 8-week-old mice. M6P inhibited epinephrine-induced uptake of ^{131}I-P-GUS in a dose-dependent manner (*A*), and epinephrine failed to increase the uptake of ^{131}I-NP-GUS (*B*). Mice received ^{131}I-P-GUS or ^{131}I-NP-GUS with ^{125}I-albumin (5.5×10^5 cpm of each) i.v. and were killed 10 min later. Epinephrine (40 nmol) and M6P (0.02, 0.2, and 2 μmol) were included in the injection solution. Dotted lines indicate the vascular space as measured with ^{125}I-albumin in the same mice. Each column represents the mean ± SE of 4–12 mice. Asterisks indicate a significant difference from P-GUS without epinephrine and M6P; *, $P < 0.05$ and ***, $P < 0.001$. Daggers show a significant difference from epinephrine-induced P-GUS uptake; †, $P < 0.05$; †††, $P < 0.001$.

experiment, and the catalytic activity of the enzyme indicates the P-GUS entered as intact enzyme.

Specificity of ^{131}I-P-GUS Uptake by Brain and Peripheral Tissues. To determine whether the epinephrine-induced transport across the BBB was mediated by the M6P receptor as it is in neonates, we examined the effects of coinjected M6P (0.02–2 μmol per mouse) on epinephrine-induced P-GUS delivery to the brain. A dose-dependent inhibition of the epinephrine-induced uptake of ^{131}I-P-GUS was observed, and 0.2 ($P < 0.001$) and 2 μmol ($P < 0.001$) of coinjected M6P completely inhibited the uptake (Fig. 5*A*), suggesting that the epinephrine-induced uptake in the adult brain is mediated by the M6P receptor. A further test of this hypothesis was carried out by the experiment shown in Fig. 5*B*, which compares the effect of epinephrine on the transport of P-GUS with that of NP-GUS, the nonphosphorylated form of the enzyme; NP-GUS is not transported across the neonatal BBB nor is it a ligand for the M6P receptor. Epinephrine induced uptake only for the M6P-phosphorylated recombinant enzyme.

We next determined whether epinephrine induced uptake by the heart and liver, two other tissues that express the M6P

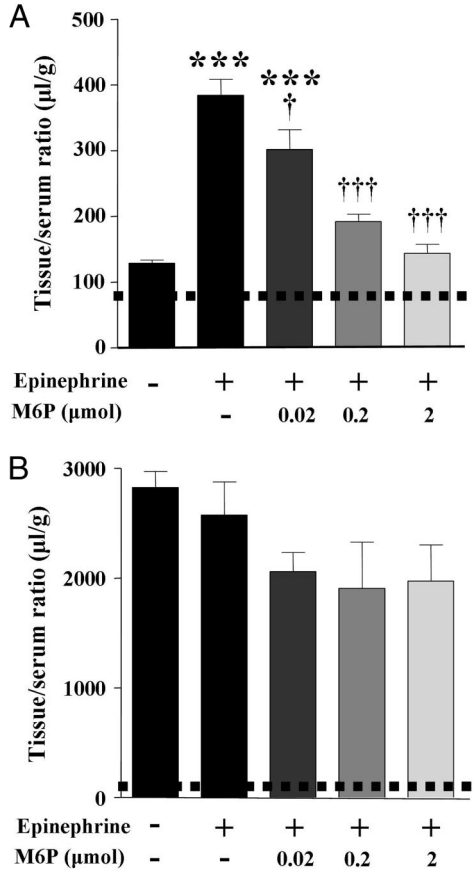

Fig. 6. M6P dependence of epinephrine-induced uptake of GUS by heart (*A*) but not by liver (*B*) in 8-week-old mice. Mice received ^{131}I-P-GUS and ^{125}I-albumin (5.5×10^5 cpm of each) i.v. and were killed 10 min later. Epinephrine (40 nmol) and M6P (0.02, 0.2, and 2 μmol) were included in the injection solution. Dotted lines indicate the vascular space as measured with ^{125}I-albumin in the same mice. Each column represents the mean ± SE of 4–12 mice. Asterisks indicate significant differences from P-GUS without epinephrine or M6P; ***, $P < 0.001$. Daggers show a significant difference from epinephrine-induced P-GUS uptake; †, $P < 0.05$; †††, $P < 0.001$.

receptor in the neonate, and whether uptake was inhibited by M6P (Fig. 6). Fig. 6*A* shows significant epinephrine-induced uptake by heart ($P < 0.001$) and that this uptake was inhibited by M6P in a dose-dependent manner (0.02 μmol: $P < 0.05$; 0.2 and 2 μmol: $P < 0.001$). By contrast, epinephrine had no effect on the quantitatively much greater delivery to liver (Fig. 6*B*), and inhibition by M6P did not reach statistical significance. In contrast to heart, where most of the delivery is mediated by the M6P receptor, most of the delivery to liver is mediated by the mannose receptor (6, 15), which is not inhibited by M6P.

Discussion

CNS delivery of lysosomal enzymes is a major obstacle to the treatment of lysosomal storage diseases in the adult (2, 5, 8). The main reason is the inability of the enzyme to penetrate the adult BBB. In our prior study, we demonstrated that saturable transport of P-GUS across the BBB in neonates is mediated by the M6P receptor (5). As a result, neonates treated with P-GUS have decreases in both CNS and peripheral tissue levels of mucopolysaccharides (2). Unfortunately, M6P receptor transport function declines with development until adults have little or no transport activity (5). As a result, treatment of adults with conventional doses of P-GUS reduces only peripheral tissue levels of storage. In this study, we found that transport of the enzyme P-GUS across the BBB could be induced in adult mice by treating them with epinephrine and that this transport was attributable to the M6P receptor, the same receptor responsible for P-GUS transport in the neonate.

We found (±)-epinephrine stimulates brain uptake of P-GUS across the BBB. The percent of the injected dose (ID) of P-GUS entering a gram of brain (%ID/g) was increased in mice treated with epinephrine, and the peak value was observed 5 min after the injection. No significant uptake of P-GUS was observed in controls, suggesting that the levels of epinephrine physiologically present in blood do not affect P-GUS transport.

Multiple-time regression analysis showed that epinephrine increased the unidirectional influx rate of P-GUS in a dose-dependent manner. The influx rate of P-GUS into adult brain after epinephrine (40 nmol) treatment was 0.29 μl/g per min. This rate is comparable to the rate seen in the neonatal mouse (0.2–0.3 μl/g per min), suggesting the transport of P-GUS in the adult BBB was restored by epinephrine to the levels that result in effective ERT in neonates.

In comparison, brain uptake of albumin as measured by the percent of ID per gram of brain was not affected by epinephrine treatment except at the highest dose (120 nmol). The initial volumes of distribution of P-GUS and albumin remained unchanged regardless of the amount of epinephrine. Therefore, epinephrine (12 and 40 nmol) did not alter the vascular space of the brain or disrupt the BBB.

A capillary depletion study revealed that, at 10 min after the coinjection of P-GUS and epinephrine (40 nmol), ≈49% of P-GUS taken up by the whole brain reached brain parenchyma and ≈51% of the P-GUS was sequestered by brain capillaries. It is unclear whether this P-GUS sequestered by the brain capillaries was permanently trapped by them or whether this represented P-GUS in transit from blood to brain.

The epinephrine-induced uptake of P-GUS in adult mice is mediated through the M6P receptor, as shown in neonates (5). The brain uptake of P-GUS induced by epinephrine was significantly reduced by the coinjection of M6P in a dose-dependent manner. Epinephrine-induced P-GUS uptake was totally inhibited by M6P at 0.2 and 2 μmol, i.e., reduced to the levels of vascular space as measured by albumin. NP-GUS, which does not have the M6P moiety on its sugar chain, did not respond to epinephrine. These results confirm that the uptake process of P-GUS at the BBB was ligand-specific and that epinephrine is a potent stimulator of P-GUS transport mediated by the M6P receptor in adult BBB. We also demonstrated that P-GUS delivered into the brain has enzymatic activity after the coinjection of nonradioactively labeled P-GUS and epinephrine (40 nmol) in MPS VII mice, indicating that the enzyme delivered was intact and biologically active.

Although both α- and β-adrenoceptors are found in brain microvessel endothelial cells (17–19), the exact mechanism by which epinephrine modulates the transport of P-GUS across the adult BBB was not elucidated in this study. Currently, there is no direct evidence that epinephrine modulates the activity of the M6P receptor itself.

Epinephrine is known to modulate endocytotic activity of the BBB, leading to changes in BBB permeability *in vivo* and *in vitro* (13, 14). Although epinephrine may stimulate fluid-phase pinocytosis, this process would be nonspecific and not receptor-mediated. The enhanced uptake of GUS is clearly M6P-specific. This finding suggests that adrenergic effects of epinephrine modify the transcytotic activity of M6P receptors, which are known to participate in the cellular trafficking of lysosomal enzymes (20, 21). Intracellular pools of M6P receptors exist in some cell types, including microvascular capillaries, that are available for translocation to the membrane surface in response to hormonal stimuli and phorbol esters (22–26). Such redistribution of M6P receptors to the capillary surface in response to

epinephrine is a plausible mechanism to explain the transport of P-GUS in adults seen in this study. Translational or transcriptional mechanisms are unlikely to explain these results as epinephrine induced its effect on transport almost immediately after injection.

Another contributing factor might be the increased availability of P-GUS to be transported from the systemic circulation into brain. Epinephrine is known to increase plasma mannose levels immediately after injection in rats (27). Because a large fraction of injected P-GUS is normally cleared by the mannose receptor in the liver (6, 15, 28), the mannose could transiently inhibit mannose receptor uptake and increase the P-GUS available to the newly expressed M6P receptors on brain capillaries.

In conclusion, the present studies indicate that epinephrine is a potent stimulator of P-GUS transport across the BBB in adult. Epinephrine-induced transport of P-GUS into adult brain parenchyma was mediated through M6P receptors and was similar to the rates seen in neonates. Thus, the pharmacological manipulation with epinephrine could be advantageous to treat CNS lysosomal storage in the adult. However, use in storage disease patients would require cautious dose adjustment or gradual administration to avoid the risk of cerebral or cardiac side effects associated with bolus administration of epinephrine.

Materials and Methods

Recombinant Human GUS Production. P-GUS was produced in overexpressing, M6P/IGF2R-deficient mouse L cells as described.[‖] The enzyme was purified from conditioned media by anti-human GUS mAb affinity column chromatography. P-GUS was eluted with 3.5 M $MgCl_2$, then desalted over Bio Gel P6 sizing resin (Bio-Rad, Hercules, CA).

Human NP-GUS was produced in insect cells by using the baculovirus system as described for mouse GUS (15). NP-GUS was purified from media from virus-infected Sf21 insect cells by anti-human GUS mAb Affi-Gel 10 affinity column chromatography by the same procedure used for P-GUS. The concentrations of both the P-GUS and NP-GUS were adjusted to 2.5×10^5 units/ml (1 unit = 1 nmol of substrate cleaved per h), and both purified enzymes were stored at −70°C. M6P specific uptake of the P-GUS- and NP-GUS-purified enzymes by human fibroblasts was 185 and 0 units/mg per h, respectively (data not shown).

Radioactive Labeling. P-GUS and NP-GUS were radioactively labeled by the Iodo-Beads method (Pierce, Rockford, IL) with $Na^{131}I$ (Perkin-Elmer, Waltham, MA). Albumin was labeled with $Na^{125}I$ (Perkin-Elmer) by the chloramine-T method. Each radioactively labeled agent was purified by Sephadex G-10 chromatography. Each agent was freshly prepared on the day of the experiment.

Animals. Male CD-1 mice from our in-house colony were studied at 8 weeks of age. The mice had free access to food and water and were maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). Studies were approved by the local Animal Care and Use Committee and done in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (Veterans Affairs Medical Center).

Drug Administration. Mice anesthetized with urethane (40%) received an i.v. injection of ^{131}I-P-GUS or ^{131}I-NP-GUS with ^{125}I-albumin (5.5×10^5 cpm of each) into the jugular vein. At 1, 3, 5, and 10 min after the injection, mice were killed, and the blood, brain, heart, and liver were collected immediately. Serum from mouse blood was isolated by centrifugation.

Effects of (±)-epinephrine (12, 40, and 120 nmol per mouse) (Sigma–Aldrich, St. Louis, MO) on the uptake of ^{131}I-P-GUS or ^{131}I-NP-GUS were examined by i.v. coinjection. Doses used were determined according to our previous study (12). To test the specificity of epinephrine-induced uptake and tissue distributions of ^{131}I-P-GUS or ^{131}I-NP-GUS, M6P (0.02, 0.2, and 2 μmol) was included in the i.v. injection.

Multiple-Time Regression Analysis. This method (29, 30) was used to calculate the blood-to-brain unidirectional influx rate (K_{in}) of radiolabeled compounds into the brain. The brain/serum ratios were plotted against exposure time estimated from:

$$Am/Cp(t) = K_{in} \cdot \left[\int_0^t Cp(t)dt \right] / Cp(t) + V_i, \qquad [1]$$

where Am and $Cp(t)$ are the cpm/g of brain and the cpm/μl of serum at time t, respectively. K_{in} was measured as the slope for the linear portion of the relation between the brain/serum ratios and respective exposure times. The exposure time was calculated as the area under the serum concentration time curve (the integral part of Eq. **1**) divided by the serum concentration at time t. The y-intercept of the line represents V_i, the distribution volume in the brain at $t = 0$.

The percents of the i.v. ID in serum (%ID/μl) and the dose taken up per gram of brain at time t (%ID/g) were calculated from:

$$\%\text{ID}/\mu l = \frac{Cp(t)}{ID} \times 100 \qquad [2]$$

$$\%\text{ID/g brain} = \left(\frac{Am}{Cp(t)} - V_i \right) \times \frac{Cp(t)}{ID} \times 100, \qquad [3]$$

where ID is the cpm injected i.v.

Capillary Depletion. To determine whether ^{131}I-P-GUS crosses the full width of the cell wall formed by the BBB, capillary depletion was performed (31). The brain was removed 10 min after i.v. injection of ^{131}I-P-GUS and ^{125}I-albumin and was emulsified in a glass homogenizer (8–10 strokes) at 4°C in a 9-fold volume of physiological buffer (10 mM Hepes/141 mM NaCl/4 mM KCl/2.8 mM $CaCl_2$/1 mM $MgSO_4$/1 mM NaH_2PO_4/10 mM D-glucose adjusted to pH 7.4). Dextran solution (molecular mass: 64–76 kDa) was added to the homogenate to a final concentration of 26%. An aliquot was centrifuged at 5,400 × g for 15 min at 4°C in a swinging bucket rotor. The pellet containing the brain microvessels and the supernatant containing the brain parenchyma were carefully separated. Results were expressed as capillary/serum and parenchyma/serum ratios, both corrected for vascular space contamination by subtracting the respective ratios for ^{125}I-albumin.

Statistical Analysis. Means are presented with their standard errors and compared by one-way ANOVA followed by Newman-Keuls multiple comparison test or by two-tailed paired t test with Welch's correction for linear regression results with the Prism 4.0 program (GraphPad, San Diego, CA).

We thank Tracey Baird for editorial assistance in the preparation of this manuscript. This study was supported by The Sanfilippo Syndrome Medical Research Foundation (W.A.B. and W.S.S.), Veterans Affairs Merit Review (W.A.B.), National Institutes of Health Grants DA019396, NS051334, and NS050547 (to W.A.B.), and National Institutes of Health Grants GM34182 and DK40163 (to W.S.S.).

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