

Evidence for Carrier-mediated Transport of Glutathione across the Blood-Brain Barrier in the Rat

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Abstract

Information on the origin of brain glutathione and the possibility of its transport from blood to brain is limited. We found a substantial uptake of ^{35}S -labeled glutathione by the rat brain using the carotid artery injection technique. The brain uptake index of glutathione with and without an irreversible gamma-glutamyl transpeptidase inhibitor, acivicin, was similar. No significant differences in the regional uptake of labeled glutathione were found in rats pretreated with acivicin. The brain uptake index of tracer glutathione was similar to that of cysteine tracer and was lower than that of phenylalanine. The transport of oxidized glutathione (glutathione disulfide) across the blood-brain barrier was not significantly different from that of sucrose, an impermeable marker. Brain radioactivity 15 s after carotid artery injection of labeled glutathione to rats pretreated with acivicin was predominantly in the form of glutathione. The *in vivo* glutathione uptake was saturable with an apparent K_m of 5.84 mM. Amino acids, amino acid analogues, and other compounds [cysteine, phenylalanine, glutathione disulfide, gamma-glutamylglutamate, gamma-glutamyl *p*-nitroanilide, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH)] did not affect glutathione transport. Our data suggest that glutathione is transported across the blood-brain barrier by a saturable and specific mechanism. (*J. Clin. Invest.* 1990; 85:2009-2013.) amino acids • brain uptake index • gamma-glutamyltranspeptidase • reduced glutathione (GSH) • oxidized glutathione (GSSG)

Introduction

Glutathione, reduced (GSH), gamma-glutamylcysteinylglycine, is an ubiquitous tripeptide in aerobic cells that plays a critical role in detoxification of peroxides and electrophilic toxins as a substrate for GSH peroxidase and GSH-S-transferase (1-4). It also may play a role in interorgan sulfur amino acid homeostasis as a storage form for cysteine (5). This latter role is based on the fact that a circulating steady-state plasma GSH level which turns over rapidly is found in rodents and

humans (5). Nearly all of the GSH in plasma is derived from GSH synthesized in the cytosol of hepatocytes and released by carrier-mediated transport (6, 7). Plasma GSH is cleared by the kidney and other organs such as intestine and lungs (8-10). Two mechanisms of clearance have been described: direct uptake of GSH by carrier-mediated transport and breakdown of GSH by gamma-glutamyltranspeptidase (GGT)¹ and dipeptidases (11).

GSH is biosynthesized from precursor amino acids by two ATP-dependent enzymatic reactions (12). Critical for the synthesis of GSH in most cells is the availability of cysteine (2). Various cell types have distinct transport systems for the uptake of cystine and cysteine (13, 14). The blood-brain barrier, along with hepatocytes, lymphocytes, and erythrocytes, lacks the cystine transport system (15). The blood-brain barrier transport of cysteine is through the L system, whereas in the other cell types mentioned, cysteine transport is mediated by the ASC system (15, 16).

GSH is abundant in the brain: both endothelium and astroglia contain millimolar concentrations of GSH (17, 18). Since cysteine and GSH levels in plasma are of the same order of magnitude, we considered that, as an alternative to plasma cysteine, plasma GSH might play an important role in brain GSH homeostasis. Brain capillaries contain GGT activity on the luminal membrane which may serve to break down plasma GSH (19). However, we considered that GSH may also be taken up intact as has been suggested in other organs that also have abundant GGT, such as intestine and lungs (8-11). Although there is some transport of peptides across the blood-brain barrier, this transport is considered not to be of physiological significance (20, 21). One previous report examined [^3H]GSH transport and showed negligible uptake (22). However, precautions to maintain GSH in the reduced form were not taken. Furthermore, we and others have found that commercially available [^3H]glycine-GSH is highly impure (23).

Methods

^{35}S -labeled isotopes (glutathione and cysteine), ^{14}C -labeled compounds (phenylalanine, sucrose, and inulin), and $^3\text{H}_2\text{O}$ were obtained from Dupont-New England Nuclear, Boston, MA. [^{35}S]GSSG (glutathione, oxidized) was prepared from [^{35}S]GSH by passing oxygen for 6 h at room temperature through a solution of GSH after initial removal of dithiothreitol (DTT) by extraction with ethyl acetate (24). Labeled

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1. Abbreviations used in this paper: BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; BUI, brain uptake index; GSSG, glutathione, oxidized; GGT, gamma-glutamyltranspeptidase.

GSSG was separated from GSH by thin-layer chromatography on preparative thin-layer plates (25), and eluted with Hepes-Ringer buffer at pH 7.4, and its purity was checked by HPLC. Unlabeled amino acids, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), glutathione, GSSG, gamma-glutamylglutamate, and gamma-glutamyl *p*-nitroanilide were purchased from Sigma Chemical Co., St. Louis, MO.

The *in vivo* intracarotid artery injection technique of Oldendorf (26) was employed for the determination of the brain uptake index (BUI) of glutathione and other compounds. Male Sprague-Dawley rats (200–300 g body weight) were anesthetized with Nembutal (50 mg/kg, *i.p.*) for 45–90 min. All experiments were carried out between 9 a.m. and 11 a.m. They were administered either acivicin in saline (2 ml/kg, 50 mM) or the saline vehicle intravenously 30 min before carotid artery injection. A mixture of 4 μ Ci of [³⁵S]GSH (195 Ci/mmol, 95–99% pure by HPLC) and 15 μ Ci of ³H₂O in 0.15–0.20 ml of Ringer-Hepes buffer/1 mM DTT at pH 7.4 was administered rapidly into the external carotid artery using a 27-gauge needle and the rat was decapitated (guillotine) 15 s later. The ipsilateral brain was removed within 30–40 s, rinsed in buffer, and blotted dry. In some experiments, hypothalamus and the frontal cortex, caudal cortex, and midbrain regions of the ipsilateral brain were dissected. The tissue samples were digested in Protosol at 50–55°C for 2 h, and the digest after discoloration with H₂O₂ was counted in Aquasol in triplicate along with quench standards. Aliquots of the injectate mixture withdrawn from the syringe were used for dose assays. BUI% was calculated (27) from the relationship $BUI\% = \frac{(^{35}\text{S} \text{ dpm in brain tissue}/^3\text{H} \text{ dpm in brain tissue})}{(^{35}\text{S} \text{ dpm in injectate}/^3\text{H} \text{ dpm in injectate})} \times 100$. In other experiments, [¹⁴C]phenylalanine (454 mCi/mmol) and [³⁵S]cysteine (600 Ci/mmol) were substituted for [³⁵S]GSH in the injectate. BUI of [¹⁴C]sucrose (5 mCi/mmol) and [¹⁴C]inulin (1–3 mCi/g) were determined in separate groups of four to six acivicin-treated rats as described above.

GSH BUIs were also determined in acivicin-pretreated rats in the presence of varying concentrations of unlabeled GSH added to the tracer in the injectate. GSH solutions of varying concentrations were prepared (1–40 mM) in Hepes-Ringer buffer per 1 mM DTT. Solutions containing higher GSH concentrations (> 1 mM) became acidic and were adjusted to pH 7.4 to avoid effects of pH on the BUI (28).

To study the inhibition of GSH uptake by amino acids and analogs, phenylalanine (1 mM), cysteine (1, 20 mM), BCH (10 mM), gamma-glutamylglutamate (10 mM), gamma-glutamyl *p*-nitroanilide (10 mM), and GSSG (20 mM) were added to the labeled injectate mixture in buffer per 1 mM DTT (except GSSG for which DTT was omitted). The pH of the solutions was adjusted to 7.4 before the addition of label and carotid artery injection. GSH BUIs were determined in a separate group of acivicin-pretreated rats with tracer GSH alone and in the presence of the above amino acids.

To determine the molecular form of the radioactivity in brain tissue, [³⁵S]GSH (50 μ Ci) was administered by intracarotid artery injection to rats pretreated with acivicin or vehicle. The rats were decapitated at 15 s and ipsilateral brain was rapidly removed. HPLC was performed on the brain homogenate after iodoacetic acid/dinitrophenyl derivatization according to the method of Fariss and Reed (29).

GGT enzyme activity in brain and kidney was determined according to the method of Szasz (30) using the assay kit from Sigma Chemical Co.

Results

BUI of GSH and other substrates in the presence or absence of acivicin pretreatment. In initial studies to validate the BUI technique, [¹⁴C]sucrose and [¹⁴C]inulin uptake was determined after carotid artery injection of the isotopes in 0.18 ml/1 mM DTT. There was only a negligible uptake of both of these compounds (BUI < 3%). There was no difference in BUIs of

sucrose and inulin between saline and acivicin-pretreated rats and no difference in BUI if DTT was omitted. Sucrose was used as an impermeable marker in all subsequent studies since its molecular weight is nearly the same as that of GSH.

The GGT activities in kidney and brain homogenates were 232 ± 18.6 and 3.5 ± 0.3 μ mol/min per g, respectively, before acivicin treatment, and decreased to below the measurable limit of the assay (< 0.1 μ mol/min per g) after administration of acivicin *in vivo*. The BUI of GSH with or without pretreatment with acivicin did not differ significantly ($22.6 \pm 2.8\%$, $n = 24$ vs. $26.6 \pm 2.1\%$, $n = 22$, respectively).

Fig. 1 shows the BUIs of [¹⁴C]sucrose, [¹⁴C]phenylalanine, [³⁵S]cysteine, [³⁵S]GSH, and [³⁵S]GSSG 15 s after intracarotid artery injection of the tracer to acivicin-pretreated rats. The BUI of GSH ($22.6 \pm 2.8\%$, $n = 24$) was lower than that of phenylalanine ($43.4 \pm 3.2\%$, $n = 5$) and significantly higher than that of the impermeable marker sucrose ($2.1 \pm 0.5\%$, $n = 6$) and similar to that of cysteine ($28.4 \pm 2.8\%$, $n = 5$). The BUI of GSSG ($3.6 \pm 1.0\%$, $n = 7$) did not differ significantly from that of sucrose (Fig. 1). The BUI values for phenylalanine and cysteine are comparable to the published results (15, 26).

Regional uptake of GSH by the brain. The BUI of tracer GSH was determined in five acivicin-treated rats in four regions of the brain: namely, hypothalamus, frontal cortex, caudal cortex, and midbrain. The mean (\pm SEM) BUIs of GSH in the above regions were $29.0 \pm 2.2\%$, $24.2 \pm 1.3\%$, $21.6 \pm 2.4\%$, and $22.6 \pm 1.6\%$, respectively. These data indicate that GSH uptake is relatively uniform throughout the brain.

Molecular form of radiolabel in the brain. Fig. 2 shows an HPLC chromatogram of radiolabel in the brain 15 s after carotid artery injection of [³⁵S]GSH in rats with or without pretreatment with acivicin. About 83% (81.9%, 84.8%) of the label was in the form of GSH in two acivicin-pretreated rats and was 64% (62.2%, 65.8%) in two rats pretreated with saline. The

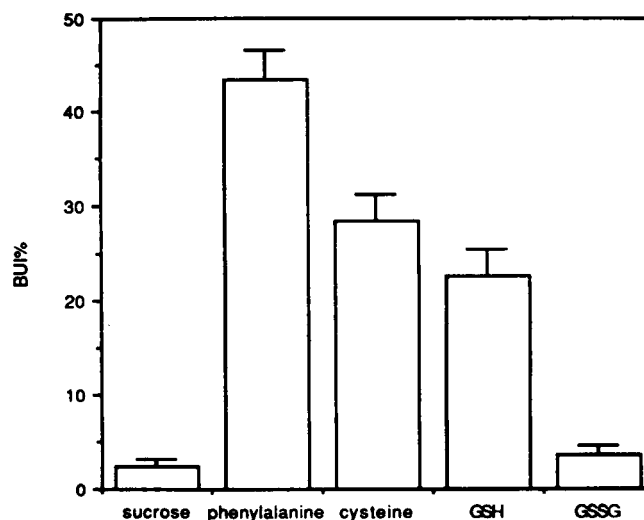


Figure 1. BUI for radiolabeled substrates in rats pretreated with acivicin. BUIs were determined 15 s after intracarotid artery injection of a mixture of 4 μ Ci (tracer only) of either [¹⁴C]sucrose, [¹⁴C]phenylalanine, [³⁵S]cysteine, [³⁵S]GSH, or [³⁵S]GSSG and 15 μ Ci ³H₂O in Ringer-Hepes buffer/1 mM DTT (except for GSSG for which DTT was omitted). Values are means \pm SEM for five to seven rats per group. The BUI for sucrose and phenylalanine were not significantly different in the absence of DTT or in rats pretreated with saline in place of acivicin (not shown).

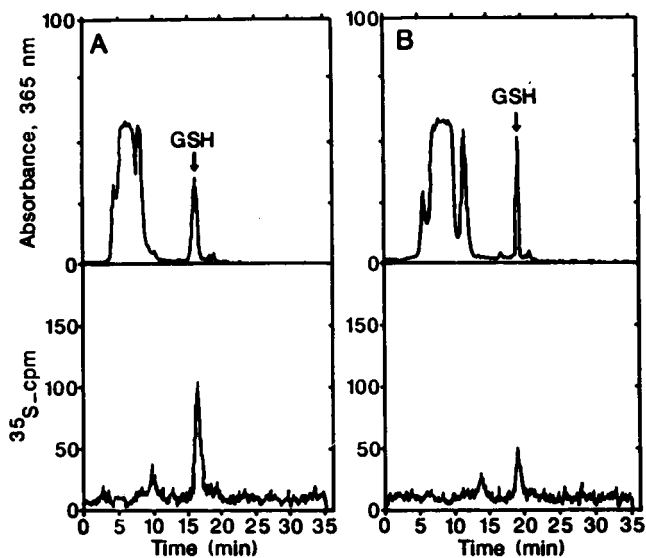


Figure 2. HPLC of rat brain homogenates 15 s after carotid artery injection of [^{35}S]GSH (50 μCi) in rats with (A) or without (B) intravenous pretreatment with acivicin. Top panels, UV absorbance at 365 nm with 0.05 absorption units as full scale; bottom panels, [^{35}S]cpm. The derivatization for HPLC was according to Fariss and Reed (29). The difference in elution time of GSH standards (arrows) in A and B is due to change in column performance.

minor peak which had a shorter retention time than that of GSH has not been characterized. In other experiments (not shown) when equivalent amounts of radiolabeled cysteine were administered, no incorporation into brain GSH was observed in 15 s as monitored by HPLC.

Saturability of GSH uptake by the brain. BUI of GSH was determined in the presence of radiolabeled GSH alone and after the addition of varying concentrations of unlabeled GSH. A significant decrease in BUI was observed when the unlabeled carrier GSH was increased from 1 to 40 mM in the injectate solution (Fig. 3 A). To convert these data to a form necessary to compute a K_m (31), the BUI_x (unlabeled GSH) for each dose was subtracted from BUI₀ (tracer dose). The results are shown in Fig. 3 B and are consistent with saturable uptake of GSH. The abscissa represents the millimolar concentration of GSH added to the injectate and the ordinate the difference between the BUI with tracer alone (BUI₀) and BUI with additional unlabeled GSH (BUI_x). A computer fit to the Michaelis-Menten equation using the simulation, analysis, and modeling (SAAM) program revealed an apparent K_m of 5.84 ± 1.92 mM for the in vivo GSH uptake. To exclude the possibility that high GSH concentrations do not cause any alterations in permeability of the blood-brain barrier, BUI of [^{14}C]sucrose was determined in the presence of 40 mM GSH and found to be unaltered (BUI 2.4%, $n = 2$).

Specificity of GSH uptake. A number of amino acids and analogues were tested to determine the specificity of GSH transport by co-administration with the tracer dose of [^{35}S]GSH. These include 1 or 20 mM cysteine, 20 mM GSSG, 10 mM gamma-glutamylglutamate, 10 mM BCH, 1 mM phenylalanine, and 10 mM gamma-glutamyl *p*-nitroanilide. In acivicin-pretreated rats ($n =$ four to six rats per group), none of these compounds significantly altered the BUI of tracer GSH determined on the same days (not shown).

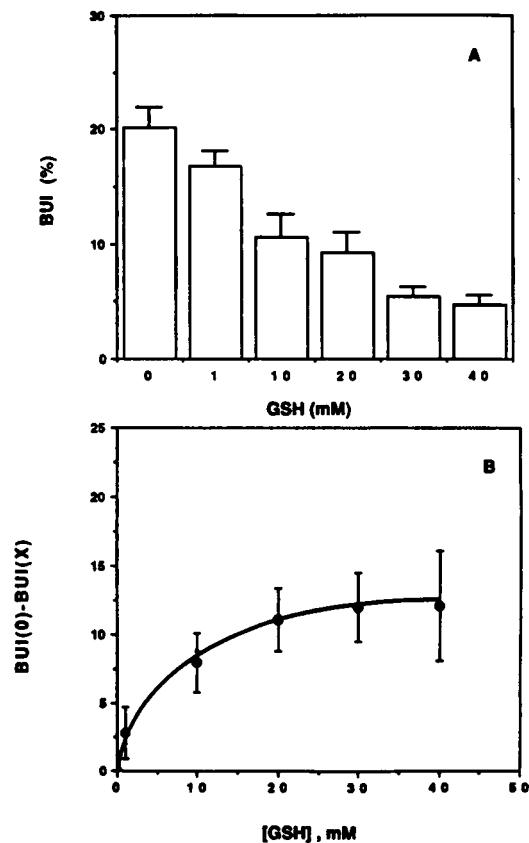


Figure 3. Saturable uptake of GSH across the blood-brain barrier in the rat. Each data point represents mean \pm SEM for 5–11 acivicin-pretreated rats. The abscissa represents the concentration (mM) of carrier GSH in the injectate (zero is tracer only) and the ordinate represents the BUI% (A) or the difference in BUI with tracer GSH alone (BUI₀) and BUI with additional unlabeled GSH (BUI_x) (B). This approach is employed as a means of depicting the data for conventional saturation kinetics. The curve represents a computer fit of the data to the Michaelis-Menten equation using the SAAM program (6). The mean (\pm SD) apparent K_m of GSH uptake is 5.84 ± 1.92 mM. Since the ordinate is dimensionless, V_{max} cannot be estimated.

Discussion

Since GSH is abundant in mammalian brain and may play a critical role in antioxidant defense, it is important to understand its regulation. We tested the hypothesis that GSH could be extracted from the lumen of brain capillaries. We used the carotid artery injection technique developed by Oldendorf (26) to assess the apparent unidirectional rapid single-pass uptake of GSH. Indeed, we found that GSH is taken up at the blood-brain barrier with efficiency comparable to that of cysteine when both are given as tracer doses. Thus, plasma GSH may be an alternative to the plasma precursor, cysteine, for providing GSH to the brain.

The uptake of GSH could not be explained by effects of DTT or acivicin on permeability of the blood-brain barrier since they did not affect sucrose uptake. The uptake appeared to exhibit saturability. The method of determination of saturability is somewhat crude due to the limitation of the in vivo experiments. Nevertheless, it appears that GSH transport exhibits a rather high K_m . However, despite the high K_m , GSH uptake was readily detected even at tracer doses, indicating

that transport can occur at plasma levels that would exist in the brain capillary lumen.

The finding of a lack of inhibition of brain GSH uptake by GSSG and other gamma-glutamyl compounds is of interest. GSH uptake in other organs such as kidney and intestine differs in that gamma-glutamyl compounds inhibit and presumably share the transport system for GSH (8–10, 32). Thus, GSH transport in these organs may simply signify a transport system for the products of transpeptidation. However, the specificity for GSH in our studies is unique and suggests a different transport system. Since GSSG did not inhibit transport of GSH and itself had a low BUI, it is possible that the previous report of inability to identify [³H]GSH transport (22) was due to its conversion to GSSG, a non-transportable form.

Although most of our studies were performed in acivicin-pretreated rats to avoid the effects of GGT, it should be noted that in the untreated rats a substantial fraction of the label was taken up in the intact form. Thus, even at tracer doses, GSH uptake can compete effectively with degradation mediated by GGT. This strongly suggests that even in the presence of GGT activity, GSH transport as the intact form may occur. Furthermore, no detectable incorporation of cysteine into GSH occurred under these experimental conditions for BUI determinations. Therefore, the labeled GSH in brain either in the presence or absence of acivicin pretreatment could not be accounted for by breakdown and de novo resynthesis of GSH. Clearly, more work will be required to precisely define the physiologic contributions of intact GSH transport vs. GGT-initiated breakdown and transport (and utilization) of cysteine at the blood–brain barrier. The ultimate determination of the contribution of the competing pathways for handling GSH, namely GSH transport and GGT, at various plasma GSH concentrations also will require assessment of the availability to the brain of the products of the GGT reaction such as cysteinylglycine and gamma-glutamylcystine. It will also be important in future work to determine the contribution of cysteine transport and GSH synthesis vs. GSH transport in determining brain GSH. However, the present studies demonstrate for the first time that under physiologic conditions transport of GSH in its intact form can occur at the blood–brain barrier.

Two aspects of compartmentation of GSH in the brain deserve comment. Choroid plexus, which constitutes only a very small portion of the brain, is reported to have a higher rate of synthesis and turnover (33) compared with that of the whole brain (34, 35). The release of GSH from choroid plexus accounts for the GSH found in the cerebrospinal fluid. Since the choroid plexus represents a very small portion of the brain, the BUI for GSH observed in our studies cannot be accounted for by uptake by the choroid plexus. Another concern about compartmentation not addressed in the present studies is whether the GSH removed from plasma is available only to endothelial cells or undergoes transcellular transport into the brain.

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