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EVIDENCE FOR TRANSCAPILLARY TRANSPORT OF REDUCED GLUTATHIONE IN VASCULAR PERFUSED GUINEA-PIG BRAIN

Berislav V. Zlokovic, Jasmina B. Mackic, J.Gordon McComb, Martin H.Weiss, Neil Kaplowitz*,[1], and Ram Kannan*

Department of Neurological Surgery and Division of Neurosurgery, Children's Hospital, Los Angeles, USC School of Medicine, Los Angeles, CA 90033

*Division of Gastrointestinal and Liver Diseases, USC School of Medicine and VA Outpatient Clinic, Los Angeles, CA 90033

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SUMMARY: Using a vascular brain perfusion model in the guinea-pig, the net uptake of [^{35}S]-GSH by the brain was found to be linear and similar in various regions during 10 min perfusion. Dual labeled [^{35}S and ^{3}H] GSH taken up by the brain had the same isotope ratio as the injected stock whether or not γ- glutamyl transferase was inhibited. Greater than 96% of brain uptake of [^{35}S]-cysteine-labeled GSH and [^{3}H]-glycine-labeled GSH were in intact form. Transcytosis of [^{35}S]-GSH from lumen into brain parenchyma was demonstrated using a capillary depletion technique. Both GSH and GSH-monoethyl ester inhibited [^{35}S]-GSH transport. Thus, we have demonstrated blood-brain barrier extraction of circulating GSH in a brain perfusion model, and the transcytosis of intact GSH into the brain parenchyma without breakdown.

Glutathione (GSH) is the most abundant acid soluble thiol in the brain (1, 2). GSH deficiency is associated with neurological changes (3,4) and a role for GSH as a neuromodulator (5, 6) has recently been proposed. Enzymes of GSH synthesis and degradation as well as transport of precursors have been demonstrated in brain (2). However, the transport system for the major precursor for GSH synthesis, cysteine, is saturated with other amino acids competing for transport, and cysteine is reported to be neurotoxic (7). Thus, we considered transport of intact GSH from plasma into the brain as an alternate source for brain GSH. We recently provided evidence for a carrier-mediated transport system for transport of circulating GSH at the blood-brain barrier (BBB) in the rat using a rapid bolus injection technique (8, 9). Whether GSH that is taken up by the brain is sequestered in the endothelium or transcytosed into brain parenchyma could not be studied in this model. We now have used a more physiological, vascular brain

[1]Corresponding author. FAX: (213) 662-7815.

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perfusion model in the guinea-pig (10) to confirm transport of intact GSH in another species and to see if capillary transcytosis into brain occurs.

MATERIALS AND METHODS

Hartley guinea-pigs of either sex (250-300 BW; 4-6 weeks) were used in perfusion experiments. The details of the vascular brain perfusion technique have been described in detail previously (10).

Pilot experiments were carried out to ascertain the suitability of the guinea-pig model for brain GSH uptake studies. Brain uptake index (BUI) after a single bolus arterial injection of tracer [^{35}S]-GSH and [^{3}H]$_2$0 was performed in 275-300 g male guinea-pigs as described earlier for rats (8). BUI of [^{14}C]-sucrose was also determined in another group of guinea-pigs of similar body weight for comparison. To determine the influence of GGT inhibition on BUI, guinea-pigs were perfused with serine borate (5 mM) for 5 min prior to the rapid bolus administration in some experiments.

Measurement of GSH uptake in the perfused brain: [^{35}S]-GSH (31 Ci/mmol); Dupont-NEN, Braintree, Mass.) in 10 mM DTT was introduced into the perfusion circuit by a Harvard slow-drive syringe at a rate of 0.2 ml/min within periods of 1 min to 10 min. [^{35}S]-GSH in the arterial inflow was found to be in reduced form by HPLC during the 10 min perfusion. In some experiments, serine-borate (5 mM) was pre-infused for 5 min before the perfusion of radiolabeled GSH and then simultaneously perfused with GSH until the end of the perfusion experiment. In a separate set of experiments to exclude degradation and resynthesis, brains from 3 guinea-pigs were perfused simultaneously with double labeled [^{35}S]-cysteine-GSH/[^{3}H]-glycine-GSH for 10 min and ratios of [^{35}S]/[^{3}H] in the perfusate and in the brain were measured. Due to the spillover and efficiency limitations in HPLC analysis of dual label, molecular forms of brain uptake were determined using tracer [^{35}S]-GSH and [^{3}H]-GSH individually in perfusion experiments (3 perfusions/label). Derivatization and HPLC analysis of brain homogenate and administered dose were carried out according to Fariss and Reed (11). Effects of addition of unlabeled GSH (1.5 mM) or glutathione monoethyl ester (70 μM) to the injectate on uptake of tracer GSH was also studied in another set of experiments. Perfusions were performed with the unlabeled GSH or its ester for 5 min followed by an additional 10 min with tracer GSH. All perfusions were terminated by severing the right common carotid artery and decapitating the animal. Radioactivity in samples of brain homogenate, vascular pellet, capillary-depleted brain (see below) and perfusate was measured in a Beckman LS-7500 liquid scintillation spectrometer. Quenching and spillover corrections were applied in dual label studies.

Transendothelial transport: In capillary-depletion experiments, the ipsilateral cerebral neocortex was quickly removed and separated into brain parenchyma and capillary pellet compartments according to the original procedure described in rats (12) and adopted to guinea-pigs in our laboratory (13). Very little contamination of supernatant by vasculature was confirmed by specific activity of vascular enzyme marker GGT whose supernatant to pellet ratio (4.3) agreed with literature values (12,13).

Calculations: Volume of distribution (V_d) for radiolabeled GSH and sucrose is calculated by the equation: V_d = [(dpm/g of tissue)]/[(dpm/ml of perfusate)], where dpm/g tissue represents [^{35}S] or [^{14}C] radioactivity per unit mass of brain homogenate, pellet or supernatant. Corrections for paracellular leak and vascular trapping within brain vascular space were made by subtracting sucrose uptake values from GSH tissue uptake values.

RESULTS

In initial studies prior to using the guinea-pig perfusion model, we determined the BUI of tracer GSH in different regions of the guinea-pig brain. BUI of tracer GSH and that of the impermeant marker sucrose were determined in six-week old male guinea-pigs. The BUIs in the caudate nucleus, hippocampus, cortex and hypothalamus were similar and the overall guinea-pig

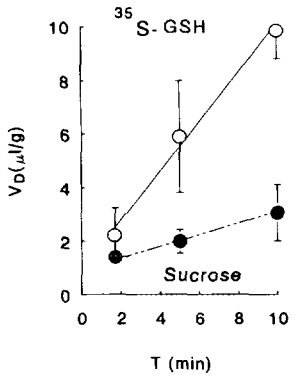

Figure 1. Uptake of [^{35}S]-GSH (4 nM) and [^{14}C]-sucrose into perfused guinea-pig brain. Perfusions were caried out in the presence of serine borate. Each point represents mean ± SE (n=3-6). T is the perfusion time.

brain BUI (13.4 ± 1.4%) was comparable to that of rats of the same age range (9). The BUIs of guinea-pig brain regions were not different when GGT was inhibited by serine-borate preperfusion (data not shown).

Uptake of tracer GSH by the perfused guinea-pig brain

Figure 1 shows the brain uptake plot obtained in perfusion experiments with either [^{35}S]-GSH (4 nM) or [^{14}C]-sucrose expressed as V_d (μl/g). These experiments were performed with serine borate to inhibit GGT. A time-dependent increase in accumulation of [^{35}S]-radioactivity in comparison to sucrose was observed with V_d values of ~ 2 μl/g brain at 100 seconds to ~10 μl/g brain at 10 min. In separate experiments (not shown), the uptake of tracer GSH in various brain regions after 10 min perfusion was similar.

Evidence for intact GSH uptake: dual labeled studies and molecular forms of uptake

To exclude the possibility that the measured radioactivity after 10 min vascular brain perfusion is due to GGT-mediated hydrolysis and resynthesis of GSH (despite use of serine borate), two sets of experiments were carried out. In the first set of 3 experiments, brains were perfused for 10 min with serine borate and tracer doses of double-labeled [^{35}S]-cysteine-GSH /[^{3}H]-glycine-GSH. The mean (± SD) ratio of [^{35}S]/[^{3}H] was determined in four regions of the brain at the termination of the perfusion. The mean ratios in hippocampus, caudate nucleus, cortex and hypothalamus were not significantly different from that of the administered dose (Table 1). In the second set, perfusions were carried out with either tracer [^{35}S]-cysteine-labeled GSH or [^{3}H]-glycine-labeled GSH with or without GGT inhibition with serine borate and molecular forms of brain uptake were determined. Figure 2 shows representative HPLC

Table 1. BRAIN REGIONAL [^{35}S]/[^{3}H] RATIOS IN DUAL LABELED GSH PERFUSIONS

	[^{35}S]/[^{3}H] ratio
Administered dose	0.217 ± 0.003
Hippocampus	0.207 ± 0.017
Caudate nucleus	0.213 ± 0.009
Cortex	0.210 ± 0.008
Hypothalamus	0.215 ± 0.014

Data are mean ± S.D. from three guinea-pigs. Experiments were carried out with initial 5 min brain perfusion with 5 mM serine borate alone and an additional 10 min perfusion along with a mixture of ~12.5 μCi [^{35}S]-GSH/60 μCi [^{3}H]-GSH in 10 mM dithiothreitol in 2 ml Krebs-Ringer buffer at pH 7.4.

chromatograms of brain homogenates after 10 min vascular brain perfusion in the presence or absence of serine borate. When [^{35}S]-GSH was used as the tracer, >96% of [^{35}S]-radioactivity was found to be in intact form under normal conditions (no serine borate) after a 10 min perfusion (Figure 2A). After10 min perfusions with tracer [^{3}H]-glycine-labeled GSH in the

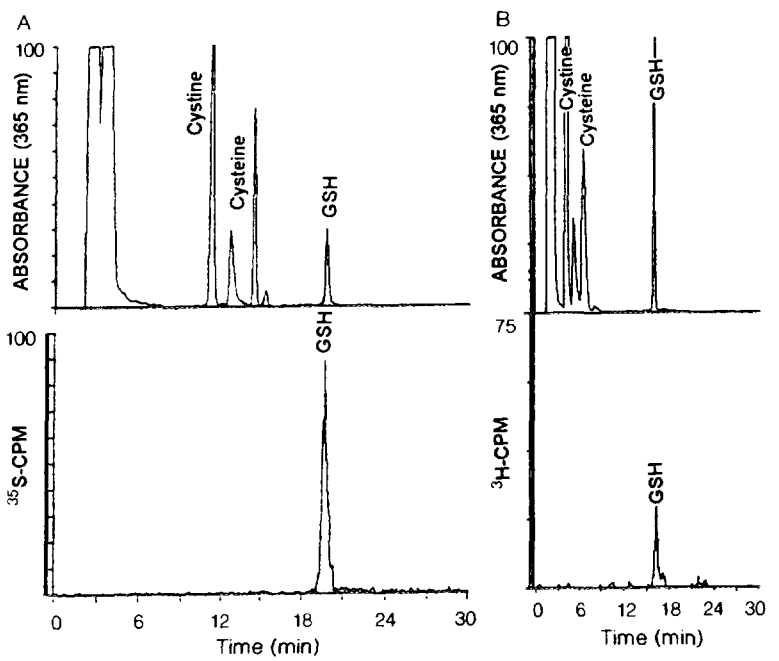

Figure 2. HPLC of neocortical homogenate after 10 min of brain perfusion with 40 nM [^{35}S]-cysteine-GSH (2A) and 40 nM [^{3}H]-glycine-GSH (2B). Top panels, UV absorbance at 365 nm with 0.05 absorption units as full scale; bottom panels [^{35}S]- or [^{3}H]-cpm. The derivatization for HPLC was according to Fariss and Reed (11). Serine borate was infused 5 min before [^{3}H]-GSH and then continuously for10 min together with [^{3}H]-GSH. The difference in retention times in Figures 2A and 2B is due to use of different columns.

presence of serine borate, again >96% of the radioactivity resided in the GSH peak (Figure 2B). Glycine elutes in void volume in this gradient system and absence of any radioactivity in the solvent front indicates absence of [^{3}H]-glycine. HPLC analyses of arterial inflow (perfusate) performed in parallel with the brain samples showed that >98.5% of the label in the arterial inflow was in GSH when either of the isotopes were used (not shown). With either [^{35}S] or [^{3}H]-GSH, label in the brain was nearly all in the form of GSH whether or not serine-borate was co-infused.

Transcapillary transport of tracer GSH

Figure 3 shows the uptake of [^{35}S]-GSH and [^{14}C]-sucrose in brain homogenate, capillary-depleted brain, and vascular pellet after 10 min brain perfusion. In both brain compartments [^{35}S]-radioactivity was significantly higher than that of sucrose, indicating cellular uptake. The major portion of the radioactivity of [^{35}S]-GSH in the brain homogenate was recovered in the capillary-depleted supernatant fraction (brain parenchyma).

Inhibitors of GSH transport

Effect of unlabeled GSH or GSH-monoethylester on the uptake of GSH in capillary-depleted brain (A) and microvessels from perfused brain (B) was examined (Figure 4). Addition of 1.5 mM GSH to the arterial inflow significantly decreased GSH radioactivity in both the capillary-depleted brain and microvessels compartments. Similarly, GSH-monoethyl ester (70 μM) also reduced GSH uptake in both compartments. It should be noted that the ester is not a substrate and does not inhibit GGT and can be viewed as an unequivocal transport inhibitor.

DISCUSSION

We have previously identified a BBB-GSH transport using the BUI technique in the rat (9). To extend these studies, we turned to the continuous vascular perfused guinea-pig brain model (10). Using this approach, a linear uptake of GSH was observed over 10 min. This duration of perfusion excludes our examining true initial rate kinetics but does permit a more sensitive approach to detect the steady-state accumulation of GSH in the brain parenchyma, which is now shown for the first time. Three additional unique features of our data should be emphasized. First, using dual labeled GSH, the isotope ratio taken up was the same as that administered. This provides very strong support for uptake of intact GSH. Had breakdown and resynthesis occurred, [^{35}S]-cysteine and [^{3}H]-glycine would have entered different pools and there would be little

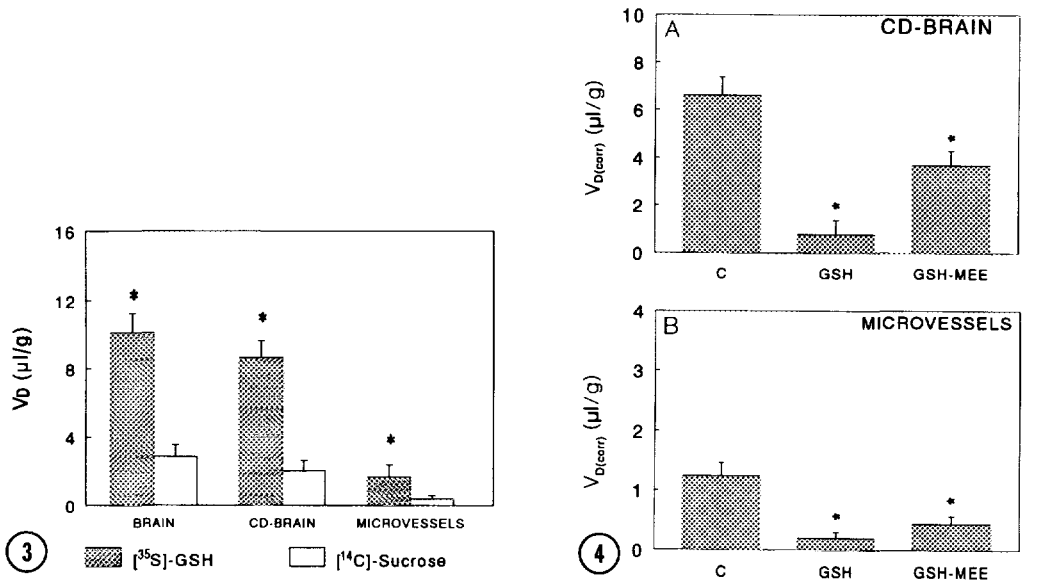

Figure 3. Compartmental distribution of [^{35}S]-GSH (4 nM) and [^{14}C]-sucrose in guinea-pig neocortical homogenate (brain), capillary-depleted (CD) brain (parenchyma) and microvessels after 10 min of brain perfusion. Values are mean ± SE (n=3-6). * indicates $p < 0.05$ vs. sucrose.

Figure 4. Inhibition of [^{35}S]-GSH uptake at the BBB in the perfused guinea-pig brain. Brains were pre-perfused with unlabeled GSH (1.5 mM) or GSH monoethyl ester (GSH-MEE) for 5 min respectively before tracer GSH was introduced and continued for the10 min of perfusion with tracer GSH. V_d for GSH in capillary-depleted (CD) brain (A) and microvascular (B) compartment was corrected for sucrose distribution. Values are mean ± SE (n=3-5). * Indicates $p<0.01$ vs. controls (C).

chance of resynthesis of GSH with the same isotope ratios as the start. Second, the capillary depletion technique has demonstrated that the bulk of the net accumulation of GSH over 10 min has been in the brain parenchyma, not the capillaries; thus transcytosis is occurring and GSH is not simply trapped in the endothelial cells. Indeed, the results support the existence of a luminal transporter for uptake and abluminal transporter for efflux of GSH in brain endothelium.

Finally, it is worth noting that although our studies demonstrate transcapillary transport of GSH in the intact brain without breakdown when GGT is inhibited, we see very little difference in GSH transport or breakdown when GGT is uninhibited. Considering the abundance of GGT at the BBB, the explanation for this seemingly surprising result remains to be fully defined. The answer may lie in the apparent abluminal and astrocyte predominance of GGT (14).

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