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TRANSPORT OF GLUTATHIONE BY RENAL BASAL-LATERAL MEMBRANE VESICLES

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Summary. Transport of glutathione was studied in membrane vesicles derived from the basal-lateral region of the plasma membrane of rat kidney proximal tubules. The integrity of the vesicle preparation was demonstrated by showing that vesicles were osmotically sensitive, with GSH uptake at equilibrium varying inversely with medium osmolality. Analysis of vesicle content by high-pressure liquid chromatography and competition experiments with glycine and cysteinylglycine confirmed that measured uptake of GSH represented transport of intact tripeptide rather than transport of degradation products. The initial rate of GSH uptake in the presence of either the sodium or potassium salt of the permeant thiocyanate anion showed that the uptake was sodium-dependent. This suggests that a GSH-Na^+ cotransport system exists in these membranes.

Several studies have shown that the kidney is very active in the extraction of glutathione, and Häberle et al.(1) have estimated that the kidney metabolizes approximately 50% of the glutathione that is released from other body tissues. The mechanism by which the kidney extracts plasma glutathione has not been completely characterized; however, the glutathione in the plasma that is filtered by the glomeruli can be degraded to its constituent amino acids in the tubular lumen by the brush-border enzymes γ-glutamyltransferase and cysteinylglycine dipeptidase. Normally, neither glutathione nor the constituent amino acids are present in the urine, but inhibition of γ-glutamyltransferase results in marked glutathionuria (2).

Only 30% of the renal blood flow is filtered through the glomerulus, yet studies have found that the renal extraction of glutathione is greater than 80% (1,3). Anderson et al. (4) showed that when rats were given a 2.5 mmol/kg body weight dose of AT-125[1], an inhibitor of γ-glutamyltransferase (5), the renal

1. Abbreviations used: AT-125, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; GSH and GSSG, reduced and oxidized glutathione, respectively; HPLC, high-pressure liquid chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propandiol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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extraction of glutathione decreased to 39%. However, Rankin and Curthoys (6) suggested that since AT-125 becomes a non-specific alkylating agent at high concentrations, other systems for the renal extraction of glutathione besides γ-glutamyltransferase may have been inhibited. They used a 50-fold lower concentration of AT-125 and found that while γ-glutamyltransferase was inhibited by 98%, the renal extraction of glutathione was still 73%. Thus, it appeared that glutathione extraction may also occur by a mechanism independent of γ-glutamyltransferase, perhaps by a paratubular transport system.

To examine this possibility, we have studied GSH transport by isolated vesicles derived from the basal-lateral region of the plasma membrane of rat kidney proximal tubules. The results show that uptake of intact GSH occurs in these osmotically sensitive vesicles and that this uptake is mediated by a Na^+-dependent process.

Materials and Methods

Phenylmethylsulfonyl fluoride, GSSG, Percoll, and 1-fluoro-2,4-dinitrobenzene were obtained from Sigma Chemical Co. (St. Louis, MO). GSH was obtained from Eastman Kodak (Rochester, NY). AT-125 (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) was a gift from Dr. Donald J. Reed. [glycine-2-^{3}H] GSH (spec. activity, 2200 Ci/mol) was obtained from New England Nuclear (Boston, MA). Nitrocellulose filters (0.45 μm pore size) were obtained from Gelman (Ann Arbor, MI). The dye-reagent concentrate for the determination of protein was obtained from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade.

Male white rats weighing 200-300 g were anaesthetized with diethyl ether and sacrificed by cutting through the diaphragm. The kidneys were immediately removed and placed in ice-cold Na^+-free 10 mM Tris-Hepes buffer, pH 7.6, containing 250 mM sucrose and 0.1 mM phenylmethylsulfonyl fluoride. The same buffer was used throughout with additions as indicated. The brush-border and basal-lateral regions of the plasma membrane were prepared according to the Percoll density-gradient centrifugation method of Scalera *et al.* (7) with the modification in buffer described above. Membrane fractions were identified by the use of marker enzymes as previously described (8). Basal-lateral membrane fractions were pooled, diluted 3-fold with Tris-Hepes buffer, and centrifuged at 100,000 g in a type 45Ti rotor at 4°C in a Beckman L8-80 ultracentrifuge. This step allowed vesicles to be concentrated and Percoll to be removed. Protein was determined by the method of Bradford (9) with bovine serum albumin as standard.

Membrane vesicles were preincubated with 0.25 mM AT-125 to inhibit GSH metabolism before uptake measurements were made (5). All solutions were made anaerobic by bubbling with prepurified nitrogen or ultrapure argon. Membrane vesicles (0.2 - 0.5 mg protein) were added to Tris-Hepes buffer containing unlabeled GSH and 2 μCi [glycine-2-^{3}H]GSH/0.3 ml incubation mixture. For experiments studying cation-dependence of uptake, sucrose was isosmotically replaced by the appropriate salt. At indicated time points, 20 μl aliquots of the incubation mixture containing 20- 50 μg membrane protein were rapidly filtered on 0.45 μm nitrocellulose filters. Filters were then washed with 4 ml of ice-cold 250 mM NaCl and were allowed to dissolve overnight in 5 ml of scintillation fluid prepared in the following proportions: 13 g

2-(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (butyl PBD) as fluor, 1.3 l toluene, 0.75 l Triton X-100, and 225 ml water. Radioactivity was then measured in a liquid scintillation counter.

HPLC measurements of GSH and its metabolites were according to the method of Reed et al. (10) which utilizes iodoacetic acid to convert thiol groups to S-carboxymethyl derivatives and 1-fluoro-2,4-dinitrobenzene to form 2,4-dinitrophenyl derivatives of amino groups. Separation was achieved on a 10 μ Ultrasil-NH_2 column (4.6 mm x 25 cm; Beckman Instruments, Norcross, GA).

Results and Discussion

The time course of GSH uptake in basal-lateral membrane vesicles was linear for the first 2 min (Fig. 1). Uptake of 1 mM GSH in the presence of a 100 mM NaSCN gradient (medium > intravesicular space) was 3.9-fold faster than uptake in the presence of a 100 mM KSCN gradient, and 4.5-fold faster than uptake in the absence of added cations. In the presence of NaSCN the initial rate of uptake was 9.84 ± 0.84 nmol GSH/min per mg protein (n=5). Similar results were obtained with NaCl and KCl, although the stimulation of uptake with Cl^- as counterion was 73% of that with SCN^- as counterion (data not shown). The time course of GSH uptake over 60 min exhibited a large overshoot

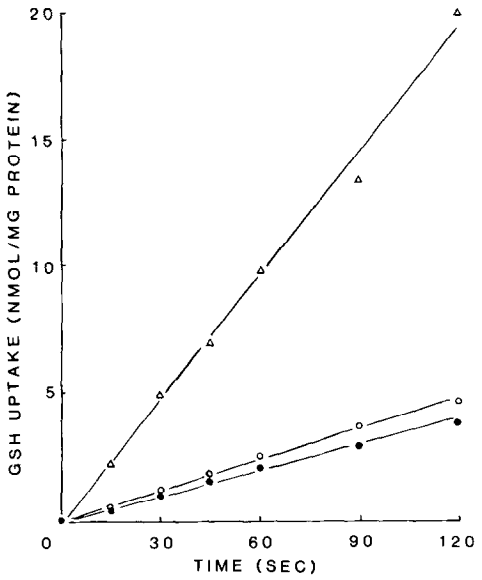
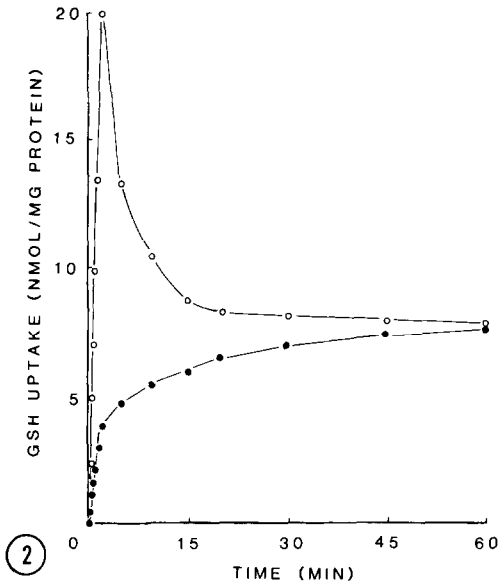


Fig. 1. Time course over linear range of GSH uptake in renal basal-lateral membrane vesicles. Membrane vesicles were incubated in the presence of 10 mM Tris-Hepes, pH 7.6, 1 mM unlabeled GSH, 2 μCi [glycine-2-^{3}H]GSH/0.3 ml incubation mixture, and either 250 mM sucrose (closed circles), 100 mM KSCN and 50 mM sucrose (open circles), or 100 mM NaSCN and 50 mM sucrose (open triangles). At each time point, 20 μl aliquots were filtered. Values are the mean of three preparations.



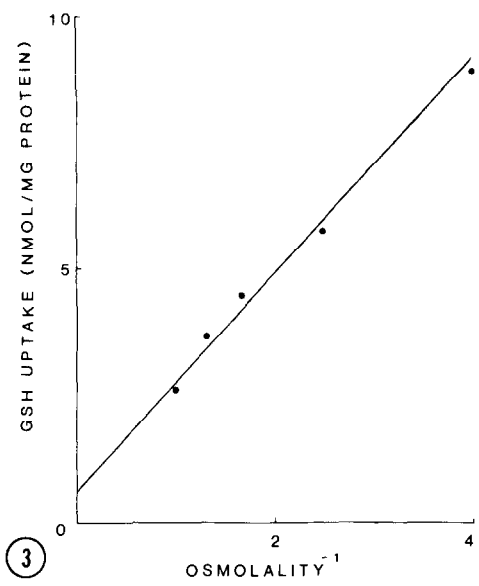


Fig. 2. Time course of GSH uptake in renal basal-lateral membrane vesicles. Membrane vesicles were incubated in the presence of 10 mM Tris-Hepes, pH 7.6, 1 mM unlabeled GSH, 2 μCi [glycine-2-^{3}H]GSH/0.3 ml incubation mixture, and either 250 mM sucrose (closed circles) or 100 mM NaSCN and 50 mM sucrose (open circles). At each time point, 20 μl aliquots were filtered. Values are the mean of three preparations.

Fig. 3. Relationship between GSH uptake and medium osmolality. Membrane vesicles were incubated in the presence of 10 mM Tris-Hepes, pH 7.6, 1 mM unlabeled GSH, 2 μCi [glycine-2-^{3}H]GSH/0.3 ml incubation mixture, and 100 mM NaSCN. Medium osmolality was adjusted with sucrose to the values indicated. Equilibrium uptake was measured after 60 min incubation by filtering 20 μl aliquots. Values are the average of two preparations.

in the presence of a 100 mM NaSCN gradient, indicating transport of GSH against a concentration gradient (Fig. 2). The overshoot reached its maximum at 2 min, and was followed by passive efflux of GSH into the medium after the Na^+ gradient was dissipated. Uptake in the presence of KSCN did not exhibit an overshoot indicating that potassium cannot support uptake of GSH.

In addition to demonstrating uptake of GSH against a concentration gradient, vesicle integrity was also shown by measuring equilibrium GSH uptake in the presence of 100 mM NaSCN at different medium osmolalities (Fig. 3). Uptake varied inversely with osmolality indicating that GSH was transported into an osmotically sensitive space. The plot did not intersect the origin at infinite osmolality. This can be due to a non-zero intravesicular volume at

infinite osmolality or to a small amount of binding of GSH to the membranes (less than 6% at 0.25 OsM).

Since the labeled GSH contains the ^{3}H in the glycyl moiety, two experiments were performed to test whether measured uptake was due to a degradation product. Vesicles were incubated in the presence of 1 mM GSH, 100 mM NaSCN, and either 5 mM glycine or 5 mM cysteinylglycine to see if they compete for transport with the labeled compound. Less than 10% inhibition of GSH uptake occurred in the presence of a 5-fold excess of glycine or cysteinylglycine indicating that little of the measured uptake was due to products of GSH degradation.

The contents of vesicles incubated for 2 min with 1 mM GSH and 100 mM NaSCN were measured by HPLC. After filtration of an aliquot of the vesicles, the filter was placed in iodoacetic acid to convert GSH to the S-carboxymethyl derivative. The filter was then removed and 1-fluoro-2,4-dinitrobenzene was added to the solution to form 2,4-dinitrophenyl derivatives. The sample was filtered to remove membrane material before HPLC analysis. Recovery of GSH by this method was approximately 90% as judged by comparison with measured uptake of radiolabeled GSH. The major peak was that representing the GSH derivative, with only traces of the GSSG, cysteinylglycine, and cystinyl-bis-glycine derivatives being detected. Therefore, the HPLC measurements and competition experiments with glycine and cysteinylglycine demonstrate that the actual species transported across the basal-lateral membrane was GSH.

The findings presented in this study indicate that a GSH-Na^{+} cotransport system is operating in the basal-lateral membrane of the renal proximal tubule and that the Na^{+} gradient can provide a driving force for the intravesicular accumulation of GSH. This transport system may account for the difference observed between total renal extraction of glutathione and the fraction of plasma glutathione filtered by the glomeruli (1,3). Since uptake was measured under anaerobic conditions to prevent oxidation of GSH to GSSG, the role of the renal thiol oxidase, which is localized on the basal-lateral region of the plasma membrane (8), cannot be presently assessed. Future studies to determine

whether GSSG is also transported across the basal-lateral membrane may help clarify this question.

Acknowledgements

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