In Vitro and ex Vivo Uptake of Glutathione (GSH) across the Intestinal Epithelium and Fate of Oral GSH after in Vivo Supplementation

Jennifer Kovacs-Nolan,† Prithy Rupa,† Toshiro Matsu,§ Mitsuru Tanaka,§ Toru Konishi,† Yusuke Sauchi,† Kenji Sato,§ Shin Ono,† and Yoshinori Mine*†

†Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1
§Division of Bioresources and Bioenvironmental Sciences, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka, Japan
*Kohjin Life Sciences Ltd., Tokyo, Japan
†Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Kyoto 606-8502, Japan
‡Graduate School of Science and Engineering, University of Toyama, Toyama, Japan

ABSTRACT: Glutathione (GSH) is the most prevalent low-molecular-weight thiol in mammalian cells and is crucial for antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole body homeostasis. GSH transport systems have been identified in the membranes of various tissues and organs, including the small intestine. However, little is known regarding GSH transport across intestinal epithelial cells. The current in vitro and ex vivo uptake study of GSH demonstrated that intact GSH can be transported across intestinal epithelial cells, suggesting that GSH uptake is not proton-dependent. It would appear that the initial uptake of GSH into cells is a rapid process. Furthermore, the visualized GSH after 60 min of transport by MALDI-MS imaging showed localization of intact GSH inside the intestinal wall. In vivo study found that ingested 13C-GSH was rapidly converted to GSSG and accumulated in red blood cells and liver, but was little present in plasma. The ingested GSH has potent nutraceutical benefits for human health to improve oxidative stress and defense in human.

KEYWORDS: glutathione, Caco-2, transport, MALDI-MS imaging, intestinal epithelial cell, LC-MS/MS

INTRODUCTION

Glutathione (GSH) is the most prevalent low-molecular-weight thiol in mammalian cells. It is crucial for antioxidant defense and the regulation of pathways essential for whole body homeostasis via catalysis by glutathione-S-transferases (GST) and glutathione peroxidases (GPx). Glutathione deficiency is associated with oxidative stress and, therefore, may play a key role in aging and the pathogenesis of many chronic diseases.

The two cytosolic enzymes, γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase, catalyze GSH synthesis from glutamate, cysteine, and glycine. This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH. Glutathione can be synthesized through its recycling after oxidation. Glutathione catabolism occurs extra-cellularly by the activity of γ-glutamyltransferase (γ-GT), which is expressed mainly on the apical surface of cells. It cleaves the γ-glutamyl moiety from GSH, transferring it to other amino acids or dipeptides and producing cysteinylglycine or cysteinylglycine conjugates. These products are further hydrolyzed by dipeptidases and taken up into the cell via specific transporters, where they can participate in GSH synthesis.

Intact GSH may be transported across both basolateral and apical surfaces, and GSH transport systems have been identified in the membranes of various tissues and organs, including the small intestine. It has been reported that oral administration of GSH could be absorbed with intact form in vivo and that dietary GSH or supplemental GSH might be an important source of circulating GSH. However, there are controversial reports regarding the bioavailability of orally administered GSH in intact animals. The uptake of intact GSH into plasma membrane vesicles in intestinal epithelial cells has been reported by Vinzenzenin et al. However, other groups have reported that orally induced GSH could be degraded by γ-glutamyl transferase and peptidases at the brush border membrane at the intestinal epithelial cell surface into amino acids of γ-glutamyl amino acid, cysteine, and glycine. In fact, a relatively large dose of orally administered GSH did not increase serum GSH levels in human subjects, and it has been concluded that dietary GSH does not alter serum GSH and this is because of degradation of intact GSH. Grattagliano et al. also found that GSH and GSH monomethyl ester (GSHE) do not affect the circulating concentration of GSH and cysteine unless very high doses are administered, but increased hepatic cysteine and GSH at lower doses because of the efficient extraction by the liver of cysteine originating from the breakdown of GSG and GSHE in the gut. After careful review of these past work on GSH uptake, we found that these controversial results might be due to differences of sample pretreatment and sampling for GSH analysis. For example, those who reported no effect of GSH intake orally conducted only plasma GSH analysis. However, we found in this study that ingested GSH is rapidly converted into GSSG.
and does not exist as free GSH in plasma, but was deposited into red cells or liver for future uses.

The objective of this work was to examine whether intact glutathione (GSH) can be transported across the intestinal epithelium and to characterize its uptake, as well as to determine the fate of orally administered GSH in animal models.

**MATERIALS AND METHODS**

**Materials.** GSH was kindly provided by Kohjin Life Science Ltd., Tokyo, Japan. 13C-GSH was synthesized by a conventional liquid-phase method via 13C-GSSG using the disulfide bond as a thiol-protecting group. l-Cystine containing 1,1′-13C isotopes (l-cystine-1,1′-13C0, Sigma-Aldrich, St. Louis, MO, USA) was converted to the corresponding N,N′-tert-butyloxycarbonyl (Boc) protected 13C-cystine using di-tert-butyl dicarbonate. The crude 13C-GSH was purified using reversed-phase HPLC on a Nakalai Tesque C18 column (COSMOSIL SC18-AR-II, 20 × 250 mm). The final product was characterized by reversed-phase HPLC using a TSKgel ODS-100S (3.0 × 150 mm; Tosoh, Tokyo, Japan) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) with a Hitachi NanoFrontier LC-MS system (Tokyo, Japan).

**In vitro Transport of GSH across Human Intestinal Epithelial Cell Monolayers.** In vitro transport experiments were performed using Caco-2 or HT-29 human intestinal epithelial cells (ATCC). Caco-2 or HT-29 cell monolayers grown in Costar transwell inserts (1.0 cm², 0.4 μm pore size) (Corning, Cambridge, MA, USA) in Eagle’s minimal essential medium (MEM) (Invitrogen, Burlington, ON, Canada) containing 2 mM α-glutamine, 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 50 μM penicillin-streptomycin (Invitrogen), and 20% FBS (HyClone) (Fisher Scientific, Ottawa, ON, Canada). The cells were seeded at a density of 0.6 × 10⁵ cells/cm², and the culture medium was changed every 2 days until confluent monolayers with transepithelial electrical resistance (TEER) values >500 Ω·cm² were obtained. TEER values were measured using a MilliCell-ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA). Cell monolayers were rinsed with Hank’s balanced salt solution (HBSS) adjusted to pH 7.4 with 10 mM HEPES, and the same buffer was added to both apical and basolateral chambers. After equilibration in buffer for 30 min, GSH (1 mM) was added to the apical chamber. Cells were incubated at 37 °C, and aliquots were removed from the basolateral chamber at the indicated time points. Apparent permeability coefficients \( P_{app} \) were calculated using the formula

\[
P_{app} = \frac{V}{A \times C_0} \frac{dC}{dT}
\]

where \( V \) is the volume in the basolateral chamber, \( A \) is the filter surface area, \( C_0 \) is the initial GSH concentration in the apical chamber, and \( dC/dT \) is the slope of the curve of basolateral GSH concentration versus time. To examine the effect of pH on GSH uptake, 1 mM GSH was prepared in HBSS buffer adjusted to pH 6.0 with 10 mMol/L MES and was added to the apical chamber.

To examine if GSH could be transported intact, Caco-2 cells were first incubated with 0.25 mM Acivicin (AT-125; Santa Cruz Biotechnology). After 2 h, media were changed by lysate buffer (Sigma-Aldrich) and subjected to further analysis. The plasma and RBC samples were immediately subjected to GSH and GSSG measurement. A reference GSH was used to confirm that GSH was not converted to GSSG during these sample pretreatments (data not shown).

**Enzymatic Detection of Transformed GSH.** GSH was quantified in the Caco-2 cells and HT-29 basolateral chamber using an enzymatic assay for the detection of GSH/GSSG, according to the manufacturer’s instructions (Oxford Biomedical Research, Rochester Hills, MI, USA). Briefly, a GSH/GSSG assay was designed to measure total, reduced, and oxidized glutathione using an Ellman’s reagent (DTNB) and glutathione reductase (GR). This method can be used to measure oxidized (GSSG) by using a specific protocol, which first scavenges all GSH with 1-methyl-2-vinylpyridinium triflate. GSH concentrations were calculated from a GSSG standard curve, and results are expressed as micromolar total GSH (GSHt) in the basolateral solution. The total GSH, reduced GSH, and GSSG in mouse plasma, red cells, and liver were quantified using the same protocol.

**Detection of GSH and 13C-GSH by Mass Spectrometry.** To confirm transport of intact GSH across Caco-2 monolayers, basolateral solutions were analyzed by LC/TOF-MS for the presence of GSH or GSH degradation products. Samples were derivatized using Thioglo3 reagent (Covalent Associates, Inc., Corvallis, OR, USA) by incubating 20 μL of basolateral solution with 20 μL of 0.2 mM reagent and incubating for 1 h at room temperature. LC-MS was carried out using a Cosmosil SC18-MS-II column (i.d. 2.0 mm × 150 mm, Nacalai Tesque, Kyoto, Japan) with a linear gradient of 0–100% methanol containing 0.1% formic acid at a flow rate of 0.2 mL/min. MS conditions were as follows: mode, negative-low, expert mode; nebulizer, 1.6 bar; dry gas, 8.0 L/min; mass range, 100–1000; hexapole RF, 200 Vpp; dry temperature, 200 °C.

**Detection of 13C-GSH transported by rat small intestine was carried out using matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) as described by Hong et al. with modifications.** Briefly, the matrix reagent and ionization enhancement (20 mg/mL 2,4,6-trihydroxyacetophenone (THAP) with 10 mM diammonium citrate) were dissolved in 50% ACN/0.1% TFA and uniformly sprayed using an ImagePrep automatic matrix sprayer (Bruker-Daltonics, Tokyo, Japan). MALDI-IMS imaging was performed using autopixel II equipped with smart beam (Bruker-Daltonics) with the following analytical parameters: mode, positive; ion source, 1.200 kV; ion source 2, 18.80 kV; lens, 7.50 kV; pulsed ion extraction, 0 ns; mass range, 120–1000 Da; laser frequency, 200 Hz; number of shots, 500 shots/spot; spatial resolution (spot distance), 50 μm; laser attenuator, 1 minimum; laser power, 100%.
Determination of $^{13}$C-GSH and $^{13}$C-GSSG by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). The concentrations of $^{13}$C-GSH and $^{13}$C-GSSG in the blood, red cells, and liver samples were determined with a slight modification of a previously reported $^{13}$C-stable isotope labeled dipeptide detection method. Sample preparation followed the method described by Nakashima et al. To prevent oxidation of GSH, all samples were flushed by N$_2$ gas at 4°C. The derivates of GSH were prepared by the same method described above using ThioGlo3 reagent. A chromatographic separation was performed on a Biosuite C18 PA-A column (Waters, Milford, MA, USA), and a mobile phase of CH$_3$CN-water containing 0.1 FA and a flow rate (0.20–0.30 mL/min) were applied. LC-MS/MS was performed as described above. Three separate replicates of $^{13}$C-GSH with different concentrations between 0.1 and 5.0 ng/mL were conducted for obtaining the calibration curve. We detected about 7% of endogenous $^{13}$C-GSH against $^{12}$C-GSH in vehicle mouse tissue samples (data not shown); therefore, we determined the actual exogenous $^{13}$C-GSH by subtracting the endogenous one. The $^{13}$C-GSSG and reduced $^{13}$C-GSH were quantified on the basis of a combination of the LC-MS/MS and an enzymatic assay.

**Statistical Analysis.** Data are expressed as means ± SEM. Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) using one-way ANOVA followed by Tukey’s multiple-comparison test. Student’s t test was used when comparisons between only two groups were carried out. Differences were considered significant when $p < 0.05$.  

### RESULTS AND DISCUSSION

Several mechanisms may be involved in the uptake and utilization of luminal GSH by brush-border membrane enzymes in the intestine, including (1) cleavage of GSH into cysteinyl glycine, $\gamma$-glutamyl amino acids, or its constituent amino acids (Glu, Cys, Gly) by $\gamma$-glutamyltransferase, followed by their transport and intracellular GSH synthesis; and (2) transport of intact GSH directly by specific transporters. GSH transport systems have been identified in the membranes of various tissues and organs, including renal cells, pulmonary alveolar...
cells, retinal pigment epithelial cells, brain endothelial cells, but less is known about its transport across the intestinal epithelium. Several studies examining GSH uptake in the intestine have been carried out using different animal species and techniques including pig and rabbit intestinal brush-border membrane vesicles (BBMV), vascularly perfused rat small intestine, and rat intestinal epithelial cells. However, little is known regarding GSH transport across intestinal epithelial cells. Here, the transport of intact GSH across human intestinal epithelial cells was examined in vitro using Caco-2 cell monolayers. GSH was detected in the basolateral transport chamber of the cell monolayers using a GSH/GSSG enzyme assay and the structure of intact GSH confirmed by mass spectrometry (LC-MS).

Caco-2 cell monolayers were treated with exogenous GSH (1 mM), and the amount of GSH present in the basolateral transport chamber of Caco-2 cell monolayers was measured at different time points using a GSH/GSSG enzyme assay. To confirm that the GSH detected in the basolateral chamber was not as a result of GSH degradation by brush-border membrane enzymes and subsequent resynthesis within the cells, cells were treated with Acivicin (AT-125) and/or buthionine sulfoximine (BSO). AT-125 can prevent GSH degradation by irreversibly inhibiting γ-glutamyltransferase, and BSO can prevent GSH synthesis through inhibition of γ-glutamylcysteine synthetase. To test whether intact GSH is transported across Caco-2 cell monolayers, cells were pretreated with AT-125 or BSO, and GSH concentration in the basolateral chamber was measured as a function of time during incubation with 1 mM exogenous GSH.

Transported GSH was measured at various time points (Figure 1), and the apparent permeability coefficients, $P_{app}$, were calculated. There was no significant difference between the concentration of GSH detected in the presence or absence of the inhibitors AT-125 or BSO, indicating that the GSH present in the basolateral chamber was likely due to the uptake/transport of intact GSH across the Caco-2 monolayers, and not GSH resynthesis. No GSH was detected from untreated control cells (cells alone). Likewise, there was no difference in the permeability ($P_{app}$) of GSH in the presence or absence of either inhibitor (Table 1). Cell viability did not appear to be affected by the treatments (data not shown). Analysis by LC-MS confirmed that the detected sample was indeed intact GSH.
Moreover, no products of GSH degradation (e.g., cysteine, cysteinyl-glycine) were observed in the transported samples (Figure 3). These results indicated that intact GSH can cross intestinal epithelial cells without degradation, in contrast to previous reports. Previously, it was speculated that intestinal epithelial cells also have the same mechanistic transport of GSH with other tissues of renal cells, pulmonary alveolar cells, retinal pigment epithelial cells, and brain endothelial cells, whereas no-one has reported clearly with direct evidence until the current work. This is the first study to demonstrate direct uptake of GSH using human intestinal epithelial cells.

To examine whether GSH uptake is pH dependent or not, we carried out a proton-dependent study using different pH gradients at apical and basolateral sides (both apical and basolateral pH values of 7.4 or an apical pH of 6.0 and a basolateral pH of 7.4, typical of proton-dependent transporters such as the di/tripeptide transporter PepT1). The amount of GSH detected in the basolateral solution was significantly higher when an apical pH of 7.4 was used when compared to an apical pH of 6.0 (Figure 4), suggesting that GSH uptake is not proton-dependent. These results are in line with previous reports that uptake of GSH into brush-border membrane vesicles was not proton-driven and occurred optimally around pH 7.5. To further characterize GSH uptake across the Caco-2 monolayers, transport was carried out in the presence of the inhibitor Probencid (PB), a known organic anion transport inhibitor and inhibitor of GSH transport in basolateral membrane vesicles. The amount of GSH detected in the basolateral solution of PB-treated cells was significantly reduced when compared to all other samples (Figure 5), suggesting transport of intact GSH.

It was notable when we used HT-29 cell monolayer, which does not express the human PepT1 transporter with the same conditions. We observed similar results (data not shown) and

**Matrix:**

20 mg/mL THAP in 60% ACN/0.1% TFA with 10 mM di-Ammonium citrate

![Figure 5. Uptake of 1 mM GSH across Caco-2 cell monolayers in the presence or absence of 0.25 mM AT-125 and 0.5 mM buthionine sulfoximine (BSO) or 1 mM Probencid (PB) after 60 min. ND, none detected. (***) P < 0.01 relative to GSH, GSH + AT-125, and GSH + AT-125 + BSO. Untreated denotes 60 min of incubation in the absence of GSH at pH 7.4.](image)

![Figure 6. MALDI-MS imaging and spectra of transported GSH using rat intestinal membrane.](image)
could confirm that intact GSH is likely being taken up or transported across the HT-29 monolayers, and not GSH resynthesis. GSH transportation across human epithelial cells is not PepT1-mediated transport. The GSH-specific transport system in human small intestine epithelial cells was reported as a Na⁺-independent transport system or Na⁺-coupled GSH transport. However, its detailed transport mechanism is still not well understood. Further characterization of GSH transport may be required, including using the Caco-2 BB (C2BBe1) cell line or examining the effect of mono- and bivalent cations on GSH uptake, and using a more sensitive detection method to detect GSH uptake at earlier time points to gain a better understanding of uptake kinetics as from the literature it would appear that the initial uptake of GSH into cells is a rapid process.

Rat intestinal epithelial tissues were treated with exogenous GSH, and transported 13C-GSH was visualized using MALDI-IMS, which has been similarly used to detect small transportable peptides in rat small intestine. MALDI-IMS imaging of GSH in the intestinal tissue sections showed clear localization of transported 13C-GSH as [GSH + H]⁺ and [GSH + Na]⁺ in the apical side of the intestinal wall after 60 min of incubation with 10 mM GSH (Figure 6). The presence of endogenous GSH in the intestinal tissue was not visualized in blank intestinal wall subjected to 60 min of incubation with KBR buffer without GSH. Neither endogenous nor transported GSSH (oxidized form of GSH), Cys-Gly, or γ-Glu-Cys was visualized in any of the intestinal samples tested (blank, 0 and 60 min GSH treated intestines). The absence of GSH on the apical surface of intestinal tissue after 0 min (actually ~10 s of incubation) of incubation with GSH would strongly suggest that the visualized GSH after 60 min of transport showed localization of intact GSH inside the intestinal wall. To our knowledge, this is the first report of visualized intact GSH in the intestinal wall and demonstrates its presence in the tissues with intact form. Matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) has been developed to visualize the target molecules in tissues, and this technique is a powerful tool for bioactive metabolomics study. However, it was limited for only biological compounds with molecular weight of 1–50 kDa, and no small bioactive peptides (<500 Da) were visualized. Recently,

Figure 7. Measurement of (A) total 13C-GSH, (B) 13C-GSSG, and (C) reduced 13C-GSH in liver, red blood cells (RBCs), and plasma of mice orally administered 10 mg of 13C-GSH. n.d., none detected. (++) P < 0.01 and (+++) P < 0.001 relative to control.
there has been much attention to the study of small di/tripeptides metabolomics, and we reported a new technique of small peptide MALDI-IMS.17 The current MALDI-IMS visualization of GSH in intestinal wall can provide new insights of intestinal absorption behavior of bioactive small compounds as foods and drugs and omics analysis of small metabolites in various tissues.

To determine the fate of orally ingested GSH, mice were supplemented with 13C-GSH (10 mg/mouse), and total 13C-GSH, 13C-GSSG, and reduced 13C-GSH in liver, RBCs, and plasma were quantified. Total 13C-GSH in liver and RBCs was significantly elevated 60 min after ingestion when compared to control mice, but no change in plasma total GSH was observed (Figure 7A). Interestingly, we found that ingested 13C-GSH was rapidly converted to GSSG and accumulated in RBCs and liver, but was not present in plasma (Figure 7B). Reduced 13C-GSH was low or negligible in all samples tested (Figure 7C), suggesting that 13C-GSH was predominantly deposited in the form of GSSG in the red cells and liver tissues after ingestion of GSH. Previously, it has been reported that dietary GSH does not increase serum GSH level after ingestion and its bioavailability is low, but increases only liver cysteine originating from the breakdown of ingested GSH in the gut.14–16 This is in line with orally ingested GSH not altering plasma total, GSSG, and reduced GSH level from the current work as reported previously. However, the previous study overlooked analyzing GSH or GSSG level from red cells and liver. In this study, we found mostly orally ingested GSH was immediately converted into GSSG and deposited into red cells and predominantly carried into liver tissues. We speculate some of the ingested 13C-GSH could be degraded during the circulation of intact GSH into γ-glutamyl amino acid, cysteine, and glycine or γ-glutamyl cysteine by endogenous enzymes. However, the current LC-MS/MS technique could not detect notable degradation of ingested GSH. This must be further clarified in the future by developing a more sensitive method. Our recent small-scale work on human subjects indicated that there was no significant change of plasma GSH after ingestion of GSH. However, the GSH contents in the protein-bound fraction of plasma significantly (P < 0.01) increased from 15 to 120 min after GSH supplementation.26 This is a very unique biological system because reduced GSH has a free thiol group (–SH) in cysteine, and an excess presence of reduced GSH in the plasma or tissues might have some harmful effects for the body due to the potential risk of reactive oxygen species (ROS) generation. If ingested GSH is deposited in circulating red cells or liver tissue as a GSSG form, it could be safer and used for detoxification in the body or liver.

GSH is the major intracellular nonprotein thiol compound and plays a major role in the protection of cells and tissue structures.12 GSH allows the detoxification of free radicals and oxygen-reactive species involved in such diseases as atherosclerosis, rheumatoid arthritis, adult respiratory distress syndrome, or reoxyenation injury; consequently, its concentration decreases in such diseases.27 Hepatic GSH concentrations have been observed in alcoholic20 and nonalcoholic30,31 liver diseases. Plasma GSH concentrations also decrease in such diseases. Therefore, supplemental ingested GSH can benefit the treatment of these diseases and increase liver GSH concentration for detoxification. The ingested GSH has potent nutraceutical benefits for human health to improve oxidative stress and defense in humans. These data indicated that there are several unknown pathways for GSH deposit in the circulating system and transfer into the liver. Further studies will unveil the exact mechanisms of orally administered GSH transport pathway and biological roles in the body.


