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Glutathione transport system in human small intestine epithelial cells

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Abstract

The present study characterizes for the first time a GSH specific transporter in a human intestinal epithelial cell line (I407). GSH metabolism is very important for the antioxidant and detoxifying action of intestine and for the maintenance of the luminal thiol-disulfide ratio involved in regulation mechanisms of the protein activity of epithelial cells. GSH level decreases have been related to physio-pathological alterations either of intestine or other organs. GSH specific transport systems have been identified in membranes of various cell types of rat, mice and rabbit. The presence of a Na^+-independent transport system of GSH is confirmed by the similar behaviour of GSH uptake time-courses when Na^+ in extracellular uptake medium was replaced with choline$^+$ or K^+ as well as by kinetic saturation and by the trans-stimulation effect on GSH uptake in GSH preloaded cells. Moreover, this transporter is activated when cations are present in extracellular medium and it is affected by membrane potential changes with an increase in GSH uptake values when membrane depolarization occurs. The present results also show a remarkable affinity and specificity of this transporter for GSH; in fact, K_m value is very low (90 ± 20 μM) and only compounds strictly related to GSH structure, such as GSH *S*-conjugates and GSH-ethyl ester, inhibit GSH uptake in I407 cells. Finally, a possible hormonal control and modulation by the thiol-disulfide status of GSH transporter activity is suggested. © 1997 Elsevier Science B.V.

Keywords: Glutathione; Transporter; Intestinal cell; (Human)

1. Introduction

GSH and GSH-related enzymes are present in different segments of human intestine and gastric mucosa [1]. GSH is involved in detoxification processes, in regulating the intracellular redox state and the activity of proteins as enzymes, transporters, receptors [2–6]. Alteration of GSH levels in certain physiological and pathological conditions have been demonstrated in several tissues. The intestine is the first organ that may be attacked by ingested toxic chemicals and carcinogens [7], and changes in intestinal GSH content have been related to an increased susceptibility to carcinogenesis [8], oxidative injury [3,9], and metal intoxication [10] as well as to typical intestinal pathologies [11]. Oral therapeutic administration of GSH has been suggested, and increases of GSH in rat intestinal mucosa after oral GSH adminis-

Abbreviations: γ-GT, γ-glutamyltranspeptidase; BME, Eagle's Basal Medium; PBS, phosphate-buffered saline; BSO, L-buthionine sulfoximine; AT_{125}, Acivicin ((L-αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxozoleacetic acid); γ-GCS, γ-glutamylcysteine synthetase; GST, GSH-*S*-transferases; GR, GSSG-reductase; GPx, GSH-peroxidase; BBMV, brush-border membrane vesicles

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tration have been measured in different studies [3,11–13]. Moreover, it has been demonstrated that dietary GSH can be absorbed intact in rat and that the upper jejunum is the principal site of GSH absorption [14,15].

Different mechanisms of GSH uptake through intestinal membranes have been suggested [3]; however, the main experimental evidences demonstrate that GSH uptake in different animal species occurs by two mechanisms:

(1) By the cleavage of GSH into its constituent amino acids (Glu, Cys, Gly) through γ-glutamyltranspeptidase (γ-GT) and a dipeptidase activity, followed by their subsequent absorption and intracellular GSH resynthesis.

(2) By specific transporters.

Both processes are independent and may occur simultaneously [16]; however, the second mechanism is less complex, faster and involves less consumption of energy. In various cell types specific GSH transport systems have been identified [12], and recently Yi et al. [17] have demonstrated that cDNA of the rat canalicular GSH transporter hybridizes with a single transcript isolated from rat brain, kidney, lung and small intestine. In particular a Na^+-independent facilitated transport for GSH and GSH *S*-conjugates in vesicles obtained from purified brush-border rabbit membranes [18] and a GSH Na^+-dependent transporter in vesicles obtained from rat basolateral membranes [19] have been characterized. The presence of these specific transporters at both intestinal cell poles may be important for the absorption of dietary and bile-derived GSH; for the maintenance of high GSH levels in intestine and in plasma; and finally for supplying other organs with GSH [12,13]. Moreover, these transporters may eliminate GSH *S*-conjugates directly into lumen or participate in their translocation from the liver to the kidney [16,20], contributing to the protective action of intestine against exogenous and endogenous noxious compounds.

A hormonal regulation of rat intestinal GSH absorption has also been demonstrated, and a physiological role of GSH in intestine has been suggested [21]. However, at present no data exist about transmembrane transporters of GSH in human intestine. The present study was undertaken to characterize GSH uptake in a human intestinal cell line (I407) derived from embryonic small intestine [22]. We employed the I407 cell line to study GSH transport in non transformed cells and in cells of small intestine, in which GSH absorption seems mainly to occur. Moreover, I407 cells are a useful experimental model to study GSH-mediated transport, considering that these undifferentiated cells do not have γ-GT, the only known membrane enzyme that can break down γ-glutamyl linkage of GSH [23]. GSH transport was studied under different experimental conditions in order to determine the presence, activity and specificity of the GSH-mediated transport system.

2. Materials and methods

[*glycine*-2-^{3}H]GSH (spec. act. 43.8 Ci/mmol) and 2-deoxy-D-[^{3}H]glucose (spec. act. 8.2 Ci/mmol) and L-[1-^{14}C]glucose (spec. act. 55 mCi/mmol) were obtained from New England Nuclear Du Pont (Boston, MA).

Nitrocellulose filters (5 μm pore size) were from Sartorius (Göttingen, Germany). The dye-reagent concentrate for the determination of protein was obtained from Bio-Rad. All other chemicals used were reagent grade and were obtained from commercial sources.

2.1. Cell culture

Intestine 407 cells derived from undifferentiated crypt cells of fetal human small intestine were obtained from ATCC (Rockwille MD, USA). Cells were grown in Eagle's Basal Medium (BME) supplemented with 15% fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For the experiments cells grown to confluence in 100-mm dishes and in 22-mm dishes were utilized.

2.2. Radiolabeled substrate uptake

Monolayers were washed twice in isotonic phosphate-buffered saline, pH 7.4 (PBS) and collected by scraping with a rubber policeman. After centrifugation ($1000 \times g$ for 10 min) the pelleted cells were resuspended in Krebs solution pH 7.4 and utilized to measure GSH, glucose uptake and protein concentration. The uptake of radiolabeled substrates was performed by a rapid filtration method as described by Vincenzini et al. [18]. GSH uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4

containing 1 mM dithiothreitol, 200 μM GSH and 0.4 μCi [^{3}H]GSH or 200 μM 2-deoxy-D-glucose and 0.8 μCi 2-deoxy-D-[^{3}H]glucose or 50 μM L-[1-^{14}C]glucose. The Krebs solution used for 2-deoxy-D-glucose uptake did not contain D-glucose and for some experiments it contained 100 μM phlorizin. In some uptake experiments, NaCl of Krebs solution was replaced isoosmotically by choline Cl or KCl or NaSCN or mannitol. In inhibition experiments cells were preincubated at 37°C for 5 min with 2 mM different compounds. In kinetic experiments GSH uptake into cells was measured after 1 min of incubation (GSH uptake was linear up to 3 min), using different concentrations of GSH (100–800 μM).

The saturation kinetic and the values of K_m and V_{max} were obtained by non-linear regression analysis after subtraction of the non-saturable component of GSH uptake.

To estimate the radiolabeled GSH absorbed on membrane surface of cells, uptake at 4°C (on ice) was also studied.

In all experimental conditions, after suitable incubation times, transport of radiolabeled compounds was stopped by the addition of 3 ml ice-cold Krebs solution (containing unlabeled GSH in experiments of GSH uptake). The mixture was then quickly filtered through a nitrocellulose filter and washed with 3 + 3 ml Krebs solution. Cell-associated radioactivity found on the filters was determined in a liquid scintillation counter. All values were corrected for radioactivity associated with filters when uptake mixture was filtered without incubation.

2.3. Unlabeled GSH uptake by HPLC analysis

Confluent cells ($\sim 2 \times 10^6$ cells) were washed with PBS and incubated at 37°C in Krebs solution pH 7.4 (7 ml) containing 1 mM dithiothreitol, 5 mM unlabeled GSH or a mixture of 5 mM Gly, Cys, Glu. In some experiments cells were preincubated for 60 min with 1 mM L-buthionine sulfoximine (BSO) and 0.5 mM Acivicin (AT_{125}), then washed with PBS and added with Krebs solution containing 5 mM unlabeled GSH.

In GSH efflux experiments cells were incubated with Krebs solution without GSH. At suitable intervals GSH uptake or efflux were terminated by aspiration of the incubation mixture followed by rapid washing with 10 ml Krebs solution for two times.

Cellular GSH was extracted with 1 ml $HClO_4$ 5%; then cells were collected by scraping, added with 50 μl of 1 mM γ-glutamylglutamate (internal standard) and sonicated for 5 s three times. The homogenate was centrifuged (12 000 × *g* for 15 min); the precipitate was solubilised in 0.5 M NaOH and used for protein determination.

The supernatant was neutralized with 2 mM K_2CO_3, centrifuged again and used to assay GSH content by HPLC as described by the method of Fariss et al. [24].

2.4. Trans-stimulation experiments

Cells cultured in 22 mm dishes were preloaded with GSH by adding 10 mM unlabeled GSH in BME, after 12 h of incubation at 37°C, medium containing GSH was aspirated and cells were washed twice in PBS and then incubated at 37°C in 1 ml Krebs solution pH 7.4 containing 1 mM dithiothreitol, 200 μM GSH and 0.4 μCi [^{3}H]GSH. At determined times (5–40 min), uptake was terminated by aspiration of the incubation mixture, followed by rapid washing with ice-cold 3 ml Krebs solution two times. Cells were then solubilized in 0.5 ml 1 M NaOH, neutralized with acetic acid and assayed for radioactivity in a liquid scintillation counter. All values were corrected for radioactivity found when uptake mixture was added without incubation. An aliquot of the NaOH extract was removed for protein determination. GSH content in cells with and without (control) preloading of 10 mM unlabeled GSH was determined by HPLC analysis as previously described.

2.5. Enzyme assay

Cells ($\sim 20 \times 10^6$) were washed twice with PBS, collected by scraping and centrifuged at 1000 × *g* for 10 min; the pelleted cells were resuspended in 2 mM Tris buffer containing 50 mM mannitol at pH 7 and lysed by sonication at 4°C. The homogenate was centrifuged at 1000 × *g* for 10 min in a microcentrifuge; the supernatant was again centrifuged at 10 000 × *g* for 25 min. The supernatant was used to assay the γ-glutamylcysteine synthetase (γ-GCS), GSH-*S*-transferases (GST), GSSG-reductase (GR) and GSH-peroxidase (GPx) activities. The precipitate was used

to assay γ-GT. The precipitate was collected, washed and centrifuged again; then the pellet, containing partially purified plasma membranes, was dissolved in the same buffer and used for γ-GT activity determination with a commercially available assay kit (Boehringer, Mannheim) using γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates. After 60 min at 37°C, the reaction mixture was centrifuged at $12000 \times g$ for 5 min and in the supernatant the *p*-nitroaniline formation was determined at 405 nm. γ-GT activity was also measured using whole cells by the method previously described, as also reported by others [25]. All other enzyme activities were assayed as described in a previous work [26].

2.6. Protein determination

The protein concentration was determined by Bradford method [27]. Bovine serum albumin (Sigma) was used as standard.

2.7. Statistical analyses

Statistical analyses were evaluated using Student's *t*-test by computer program on the Philips PR07BM743. A difference of $P < 0.05$ was considered significant.

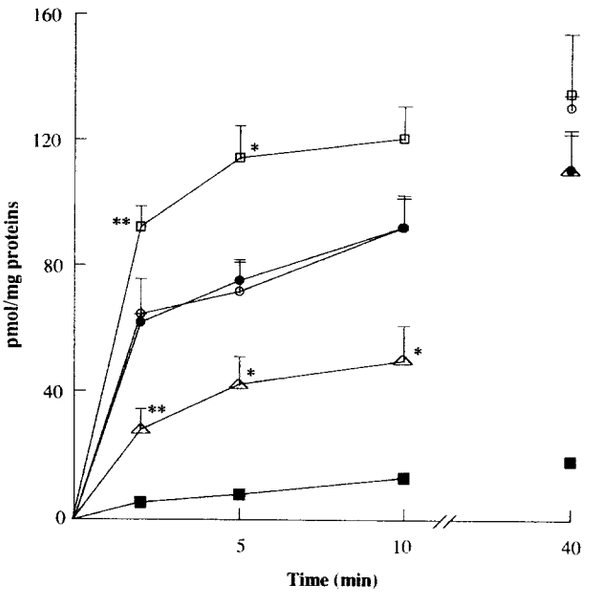

Fig. 1. Time-course of GSH uptake into I407 cells. GSH uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing 200 μM GSH and 0.4 μCi [^{3}H]GSH (○), 150 mM NaCl was replaced by 150 mM choline Cl (●), or 150 mM KCl (□) or 300 mM mannitol (△). L-Glucose uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing 50 μM L-[^{14}C]glucose (■). The data are the means ± S.E.M. of four experiments performed in triplicate. * $P < 0.05$; ** $P < 0.005$ when compared to values measured in presence of 150 mM NaCl.

3. Results

3.1. GSH levels and GSH-related enzymes in I407 cells

GSH intracellular levels and specific activities of GSH-related enzymes were measured to verify the effective occurrence of GSH metabolism (Table 1). Table 1 shows that in these cells are present both the regulator enzyme of intracellular GSH synthesis, γ-GCS, and the enzymes involved in the detoxifying and reducing action of GSH, i.e., GST, GPx and GR. Membrane enzyme activity, γ-GT, was also measured, either using whole cells or partially purified membranes; no activity was detectable. As control of membrane integrity and purification we also measured the specific activity of a typical membrane enzyme, alkaline phosphatase, which shows an enrichment factor of about 13-fold when compared to the specific activity in total cell homogenate. The

Table 1
GSH levels and GSH-related enzymes in I407 cells

GSH	γ-GCS	GST	GR	GPx
21.5 ± 1.0	0.16 ± 0.014	0.016 ± 0.002	0.016 ± 0.002	0.02 ± 0.003

GSH levels are measured by HPLC analysis and expressed as nmol/mg protein. Specific activities of enzymes are expressed as μmol/mg protein per min. Data are means ± S.E.M. of three experiments performed in duplicate. γ-GCS, γ-glutamylcysteine synthetase; GST, GSH-*S*-transferases; GR, GSSG-reductase; GPx, GSH-peroxidase.

lack of γ-GT allowed us to study GSH uptake without the interference of this enzyme activity.

3.2. Transmembrane-mediated transport of GSH in I407 cells

Fig. 1 shows that GSH was taken up into the cells in a time-dependent manner and the time-course of GSH uptake was different from that of L-glucose, which showed a typical diffusional behaviour. The GSH uptake time-course did not change when a similar Na^+ or $choline^+$ gradient was present in extracellular uptake media (Krebs solution modified) indicating the absence of a Na^+-dependent transporter. The replacement of extracellular Na^+ with an equal K^+ concentration increased GSH uptake values by about 50% for short incubation times; on the contrary, when Na^+ concentration was substituted with mannitol, very low values of GSH uptake were detected. The GSH uptake values measured in the presence of K^+ or mannitol were statistically different from those obtained in the presence of Na^+ in Krebs solution. The equilibrium values of GSH uptake were reached after 40 min of incubation and were approximately the same, indicating that different experimental conditions did not alter cellular volume.

The subsequent experiments to characterize GSH-mediated transport were performed in Krebs solution, in the presence of extracellular Na^+ gradient as it occurs in physiological conditions. To verify the aspecific binding of GSH on membrane surface of cells, GSH uptake experiments were performed at 4°C; the values of labeled GSH measured after different incubation times were similar and very low when compared to those obtained at 37°C, indicating negligible GSH binding (data not shown).

Moreover, GSH transport into I407 cells was confirmed by GSH uptake time-course obtained by HPLC measurements of intracellular GSH levels after different times of incubation with unlabeled GSH in Krebs solution (Fig. 2). Other experiments were performed to confirm the uptake of intact GSH by a transport system and to exclude intracellular increases of GSH levels due to its extracellular degradation and the subsequent uptake of its three constituent amino acids followed by GSH intracellular resynthesis. Effectively, no difference in GSH uptake time-course values was measured by HPLC analysis whether cells were preincubated for 60 min in Krebs solution with or without an addition of 1 mM BSO (a specific inhibitor of γ-GCS) and 0.5 mM AT_{125} (a specific inhibitor of γ-GT). Moreover, no increase in intracellular GSH levels was measured at different time intervals when uptake solution contained only a mixture of 5 mM Gly, Cys, Glu (data not shown). These experiments indicated no involvement of γ-GT and/or endogenous metabolism of GSH in the time-course of GSH uptake. Finally, GSH efflux from cells was also tested by HPLC analysis, incubating cells up to 90 min in Krebs solution; no change in the intracellular content of GSH was found (data not shown).

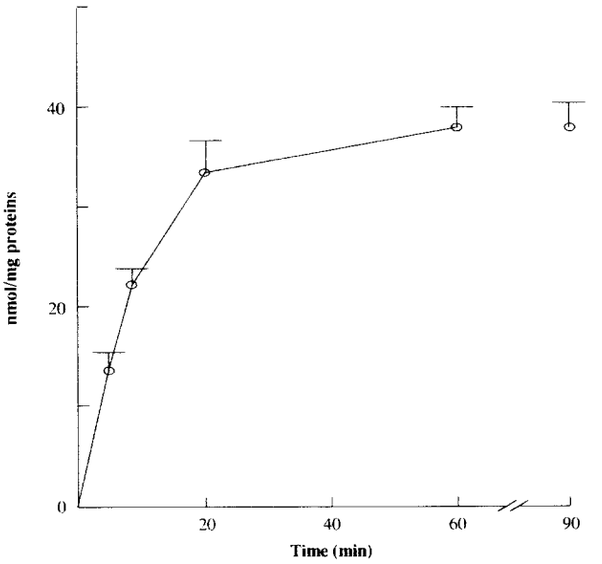

Fig. 2. Time-course of GSH uptake measured by HPLC analysis into I407 cells. GSH uptake was carried out at 37°C in 2×10^6 cells in Krebs solution pH 7.4 containing 5 mM unlabeled GSH. The values are the means ± S.E.M. of three experiments after substraction of GSH intracellular content measured in cells incubated in Krebs solution without GSH.

Since no metabolite transport system in I407 membranes was identified, the time-courses of Na^+-dependent and Na^+-independent transport of 2-deoxy-D-glucose (2-D-Glu) were measured (Fig. 3). The presence of an active transport of 2-D-Glu by specific inhibition of phlorizin was confirmed. In fact, 2-D-Glu uptake decreased significantly in the presence of phlorizin or when Na^+ was substituted with K^+. The time-courses of GSH and 2-D-Glu uptake in Krebs

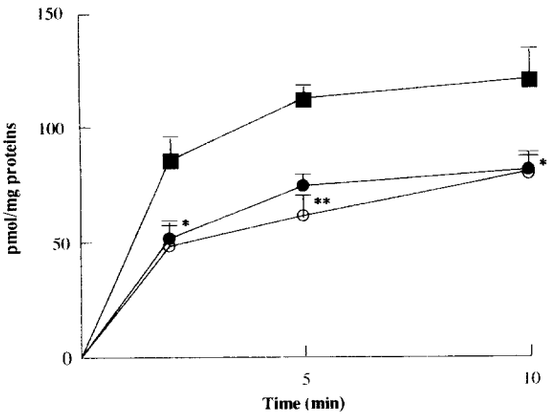

Fig. 3. Time-course of 2-deoxy-D-glucose uptake into I407 cells. 2-D-Glu uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing 200 μM 2-D-Glu and 0.8 μCi 2-D-[^{3}H]Glu without (■) and with 100 μM phlorizin (●). 150 mM of NaCl was replaced by 150 mM KCl (○). The values are the means ± S.E.M. of four experiments performed in triplicate. $^*P < 0.05$; $^{**}P < 0.005$ when compared to values measured in presence of 150 mM NaCl.

solution were similar: both reached the highest uptake values during the first minutes of incubation, confirming the presence of a mediated transport system for GSH.

3.3. Effect of membrane potential on GSH uptake in I407 cells

Table 2 shows that GSH uptake value at 5 min of incubation was affected by changes in membrane potential. In fact, a significant increase in GSH uptake (about 60%) was measured when extracellular Na^+ was replaced with K^+, which is able to depolarize the plasma membrane of these cells at high extracellular concentrations [22]. On the contrary, GSH uptake values decreased (about 35%) when anion Cl^- was substituted with the more permeant anion SCN^-, which may hyperpolarize the electrical membrane potential [28,29]. The equilibrium values measured after 40 min of incubation did not vary in different experimental conditions.

Table 2
Effect of membrane potential on GSH uptake into I407 cells.

Incubation time (min)	Uptake (pmol GSH/mg protein)		
	NaCl (control)	KCl	NaSCN
5	75 ± 8	120 ± 5 [a]	50 ± 3.5 [a]
40	140 ± 23	160 ± 10	170 ± 20

GSH uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing 200 μM GSH and 0.4 μCi [^{3}H]GSH (control) or in Krebs solution modified by substitution of 150 mM NaCl with 150 mM KCl or with 150 mM NaSCN. The data represent means ± S.E.M. of three experiments performed in triplicate.
[a] $P < 0.05$ when compared to controls.

3.4. Concentration dependence of GSH uptake

To test whether the GSH uptake in I407 cells reflects a transport-mediated process, the initial rate of uptake was determined at different GSH concentrations. Since linearity with incubation time was observed up to 3 min, GSH uptake was measured after 1 min of incubation in the range of substrate concentrations (100–800 μM). The results show a diffusional component at the highest GSH concentrations (Fig. 4). Saturation kinetic for different substrate concentrations was obtained by subtracting the diffusional component. This indicates the presence of a single GSH mediated transport system.

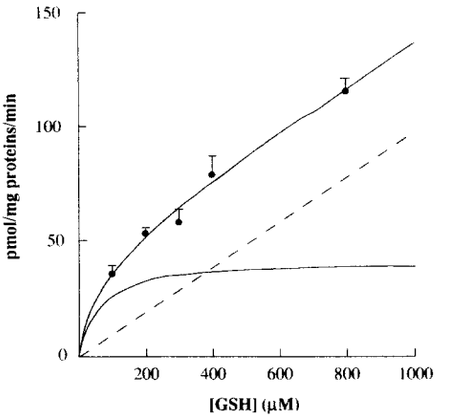

Fig. 4. Effect of varying GSH concentrations on the initial rate of GSH uptake into I407 cells. GSH uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing different concentrations of GSH and [^{3}H]GSH (100–800 μM) (●). The dashed line is a graphical estimate of the non-saturable component of GSH uptake. The continuous line shows the saturation kinetic obtained by non-linear regression, i.e. by fitting the equation $V = (V_{max}[S]/K_m + [S]) + K_d[S]$ to the experimental data. The data are the means ± S.E.M. of three experiments performed in triplicate. $K_m = 90\ \mu M \pm 20$; $V_{max} = 50 \pm 5$ pmol/min per mg protein. $K_d = 0.09$ pmol/min per mg protein.

3.5. Trans-stimulation of GSH uptake in I407 cells

A typical trans-stimulation experiment with cells preloaded with unlabeled GSH was performed to confirm the presence of a carrier mediated transport of GSH [18]. Table 3 shows that an increase of intracellular GSH (about 200%) stimulated extracellular GSH uptake, with an increase of approximately 50% after 5 min of incubation as compared to control. This activation effect due to the acceleration of external substrate transport into cells by internal substrate is interpreted as a counter-exchange of the external with the internal substrate by transporter. This activation effect on GSH uptake was not due to volume variation, considering that the equilibrium values measured after 40 min of incubation were similar.

3.6. Effect of GSH-structurally related compounds on GSH uptake in I407 cells

To verify the specificity of this transport system, the effect of different structurally related compounds on GSH uptake was studied. A significant inhibitory effect on the rate of GSH uptake after 5 min of incubation was measured only in the presence of GSH *S*-conjugates and GSH-ethyl ester, while GSSG was responsible for a significant activation effect (Table 4).

To exclude a possible GSH active transport, the effect of 1 mM ouabain on GSH uptake was studied; no alteration in GSH uptake values was measured, nor was any direct influence of 1 mM ATP on transporter activity observed (data not shown).

Table 3
Trans-stimulation of GSH uptake into I407 cells

	GSH (nmol/mg protein)	Uptake (pmol GSH/mg protein)	
		5 min	40 min
Control	23.3 ± 1.5	240 ± 35	680 ± 100
Preloaded cells	70 ± 12 [a]	380 ± 40 [a]	700 ± 40

Cells with and without (control) preloading of 10 mM unlabeled GSH were employed. GSH uptake was carried out in Krebs solution pH 7.4 containing 200 μM GSH and 0.4 μCi [^{3}H]GSH. Unlabeled GSH content in cells preloaded with and without GSH was measured by HPLC analysis. Values are means ± S.E.M. of four experiments performed in triplicate.
[a] $P < 0.05$ when compared to controls.

Table 4
Effect of structurally related compounds on GSH uptake into I407 cells

Addition	GSH uptake (pmol/mg protein)
None (Control)	80 ± 4.0
Glycine	75 ± 10.0
Glutamic acid	73 ± 8.0
Cysteine	70 ± 10.0
γ-Glu-Gly	70 ± 9.0
Gly-Gly-Gly	70 ± 9.0
Gly-sarcosine	77 ± 10.0
PNB-SG	50 ± 5.0 (−38) [b]
E-SG	45 ± 4.4 (−45) [b]
GSSG	107 ± 6.4 (+34) [a]
GSH-ethyl ester	45 ± 3.3 (−45) [a]
Probenecid	80 ± 6.0
Ophthalmic acid (γ-Glu-α-aminoisobutyrate-Gly)	70 ± 6.0

GSH uptake was carried out at 37°C in 8×10^5 cells in Krebs solution pH 7.4 containing 200 μM GSH and 0.8 μCi [^{3}H]GSH and in the absence or presence of 2 mM different compounds after 5 min of incubation. Data are the means ± S.E.M. of three experiments performed in triplicate. The values in parentheses are the percentage changes of GSH uptake compared with control value. PNB-SG, *S*-*p*-nitrobenzyl-GSH; E-SG, *S*-ethyl GSH.
[a] $P < 0.05$, [b] $P < 0.005$ when compared to controls.

4. Discussion

The present study shows for the first time a GSH specific transport system in human intestinal cells and it defines some characteristics of its transport activity. Moreover GSH metabolism in I407 cells has been confirmed measuring the specific activity of GSH-related enzymes. γ-GCS and GPx activity values are similar to those measured in human ileum and colon, while GST and GR values are lower [1,11]. Various data confirm the existence of mediated transport of GSH into I407 cells: (1) The fast uptake at short incubation time similar to that observed in glucose uptake by Na^+-dependent and independent transport. (2) The behaviour of the time-course of GSH uptake obtained by HPLC measurements, performed also in experimental conditions that exclude

the involvement of γ-GT and γ-GCS activity. (3) The saturation kinetic obtained after subtraction of the diffusional component, which is often observed in amino acid and peptide transport measurements [30]. (4) The trans-stimulation effect on GSH uptake in GSH-preloaded cells, similar to that previously measured in rabbit brush-border membrane vesicles (BBMV) [18]. In rat hepatocytes an analogous observation was also utilized to confirm the presence of GSH mediated transport [31]. (5) Inhibition of GSH uptake by GSH-related compounds, as also previously demonstrated in rabbit BBMV [18].

These results indicate that GSH uptake in I407 cells may occur only by a facilitated Na^+-independent transport system, considering the lack of γ-GT activity and the similar behaviour observed in the time course of GSH uptake when extracellular Na^+ is substituted with choline$^+$; this conclusion is also supported by the different behaviour of GSH uptake with respect to that of glucose in the presence of extracellular K^+.

As regards γ-GT activity, our data may be in agreement with Meister et al. [32] who measured very low γ-GT activity in crypt cells of the rat jejunal mucosa. There are also other findings which show that the maximum expression of γ-GT activity is related to the differentiation of enterocytes [33]. Indeed, some characteristics of both glucose transporters also seem related to enterocyte differentiation [34]. The fact that I407 cells are undifferentiated could also explain the lack of a Na^+-dependent GSH transport system in these cells; while this system has been identified in rat intestinal basolateral membranes [19]. The presence of a single GSH transport system in I407 cells is further confirmed by the kinetic analysis. Moreover, GSH transport in I407 cells is increased when cations are present in the medium, but an activation effect by a specific cation gradient is excluded, considering that all cations used in extracellular medium increase GSH uptake with respect to uptake values obtained when cations are replaced with mannitol. The increase of the GSH transport rate by extracellular cations may be explained considering that at physiological pH values GSH is negatively charged, and extracellular cations may neutralize this negative charge by cotransport with GSH. It is also possible that cations interact directly with the transporter and increase its transport activity by inducing an optimal conformational state, as we suggested in a previous work [29]. These results are in part different from findings obtained in rabbit BBMV [16,29], in which GSH uptake values are similar in the presence of Na^+ or K^+ in extravesicular medium and higher than those measured in the presence of choline$^+$. Probably these different findings are related to membrane potential changes that occur in I407 cells and not in rabbit BBMV, when Na^+ in uptake medium is replaced with K^+ or choline$^+$. However, GSH transport systems characterized in rabbit BBMV, in rat liver and kidney cell membranes are also remarkably affected by membrane potential [29]. In I407 cells membrane depolarization, with a decrease in intracellular negative charge, increases GSH uptake values, while a membrane hyperpolarization obtained using thiocyanate is responsible for a GSH uptake decrease. It is interesting to compare our data with those showed in isolated rat hepatocytes, where, under similar experimental conditions, opposite results are obtained regarding GSH efflux: in fact, the increase in concentration of medium K^+ or choline$^+$ instead of Na^+ causes a parallel fall both in membrane potential and GSH efflux, while hyperpolarization of hepatocytes with lipophilic anion thiocyanate is associated with significant increased efflux [28,31]. Since I407 cells retain receptors for intestinal secretogogues [22,35] that may change transmembrane potential by alteration of K^+ membrane conductance and cytosolic Ca^{2+} content, it is possible to hypothesize an hormonal control mechanism of GSH transport in these cells. Indeed, both a stimulation of GSH absorption in rat small intestine by α-adrenergic agonists mediated by Ca^{2+}-calmodulin dependent mechanisms [21] and an hormonal control of GSH efflux from hepatocytes mediated by changes in membrane potential [36] have been demonstrated.

The present results also indicate a remarkable affinity and specificity of this transporter for GSH; in fact, K_m value is very low if compared either to other values measured in membrane vesicles or to intact cells for GSH transport systems and other peptides [16,37]. Moreover the high specificity of this transporter for the entire structure of GSH is confirmed by the GSH uptake inhibition obtained only by compounds strictly related to GSH, such as GSH-*S*-conjugates and GSH-ethyl ester. These data are

partly in agreement with those previous obtained on the GSH transport system in rat intestinal and renal BBMV [29,38] and on the GSH low-affinity transport system present in rat liver canalicular membrane vesicles [37]. On the contrary, this inhibition picture differs greatly from that shown in intestinal or renal basolateral membrane vesicles [39,40] and in rat liver sinusoidal membrane [31]. These data also suggest the possibility that GSH-*S*-conjugates cross human intestinal membrane by the same GSH transport system as previously demonstrated in rabbit intestinal membranes [29] (other studies should be performed to confirm this). Recently, a GSH-*S*-conjugate mediated transport in Caco-2 cells (human colon adenocarcinoma cell line) has been identified [20]. An apparently contrasting effect regards the increase of GSH uptake value induced by GSSG (a GSH homoconjugate), which has been shown to be unable to affect GSH transport activity in rabbit intestine [29]; this result may be explained by hypothesizing the requirement of disulfide groups and/or the occurrence of thiol–disulfide exchange reactions in the transporter in order to increase its transport activity. Recent findings show that the bidirectional sinusoidal GSH transport in hepatocytes is modulated by the thiol-disulfide status, with a GSH efflux stimulation by dithiothreitol (thiol reduction) and a GSH uptake stimulation by cystine (thiol oxidation) [41].

It would be interesting to investigate further this GSSG effect in human GSH intestinal transport, considering that GSSG is present in a variety of foods [42] and that it may enter the body by the diet. Moreover, both GSH and GSSG are important in the maintenance of the luminal thiol-disulfide ratio, which is involved in regulating the transport and protection activity of epithelial cells as well as the turnover of mucus and enterocytes [16,43]. In conclusion, the characterization of GSH metabolism and transport in I407 cells may contribute to clarify the role of GSH in human intestine, which through the GSH interorgan cycle may be correlated to the liver and kidney in its detoxifying and antioxidant action. Moreover, the presence of GSH transporter in human epithelial intestinal cells may confirm the possible efficacy of GSH oral therapy to increase GSH levels in intestine and in other organs, in particular for the treatment of disease processes involving oxidative or electrophilic damage.

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References

[1] C.P. Siegers, H. Bose-Younes, E. Thies, R. Hoppenkamps, M. Younes, J. Cancer Res. Clin. Oncol. 107 (1984) 238–241.
[2] A. Meister, In: D. Dolphin, R. Poulson, O. Avromonic (Eds.), Coenzymes and Cofactors. Glutathione Chemical, Biochemical and Medical Aspects, vol. 3, part A, Wiley, New York, 1989, pp. 367–474.
[3] J. Martensson, A. Jain, A. Meister, Proc. Natl. Acad. Sci. USA 87 (1990) 1715–1719.
[4] D.M. Ziegler, Annu. Rev. Biochem. 54 (1985) 305–329.
[5] T. Terada, H. Maeda, K. Okamoto, T. Nishinaka, T. Mizoguchi, T. Nishihara, Arch. Biochem. Biophys. 300 (1993) 495–500.
[6] S. Rigacci, T. Iantomasi, P. Marraccini, A. Berti, M.T. Vincenzini, G. Ramponi, Biochem. J. 324 (1997) 791–796.
[7] G.J. Mulder, J.M. Tekoppele, In: H. Sies, B. Ketterer, (Eds.), Glutathione Conjugation, Mechanisms and Biological Significance, Academic Press, New York, 1988, pp. 357–389.
[8] C.P. Siegers, D. Riemann, E. Thies, M. Younes, Cancer Lett. 40 (1988) 71–76.
[9] F.J. Kelly, Br. J. Nutr. 69 (1993) 589–596.
[10] J.P. Keogh, B. Steffen, C.P. Siegers, J. Toxicol. Environ. Health 43 (1994) 351–359.
[11] T. Iantomasi, P. Marraccini, F. Favilli, M.T. Vincenzini, P. Ferretti, F. ToneIIi, Biochem. Med. Metab. Biol. 53 (1994) 87–91.
[12] T.Y. Aw, G. Wierzbicka, D.P. Jones, Chem. Biol. Interact. 80 (1991) 89–97.
[13] F. Favilli, P. Marraccini, T. Iantomasi, M.T. Vincenzini, Br. J. Nutr. 78 (1997) 293–300.
[14] T.M. Hagen, G.T. Wierzbicka, A.H. Sillau, B.B. Bowman, D.P. Jones, Am. J. Physiol. 259 (1990) G524–G529.
[15] D.P. Jones, In: L. Packer (Ed.), Methods in Enzymology, Academic Press, New York, 1995, pp. 3–13.
[16] M.T. Vincenzini, F. Favilli, T. Iantomasi, Biochim. Biophys. Acta 1113 (1992) 13–23.
[17] J.R. Yi, S. Lu, J. Fernandez-Checa, N. Kaplowitz, J. Clin. Invest. 93 (1994) 1841–1845.
[18] M.T. Vincenzini, F. Favilli, M. Stio, T. Iantomasi, Biochim. Biophys. Acta 1073 (1991) 571–579.
[19] L.H. Lash, T.M. Hagen, D.P. Jones, Proc. Natl. Acad. Sci. USA 83 (1986) 4641–4645.
[20] Y. Gietl, S. Vamvakas, M.W. Anders, Am. Soc. Pharmacol. Exp. Ther. 19 (1991) 703–707.

[21] T.M. Hagen, C. Baj, D.P. Jones, FASEB J. 5 (1991) 2721–2721.
[22] T. Yada, Y. Okada, J. Membr. Biol. 77 (1984) 33–44.
[23] M.E. Anderson, A. Meister, Proc. Natl. Acad. Sci. USA 80 (1983) 707–711.
[24] M.W. Fariss, D.J. Reed, Methods Enzymol. 143 (1987) 101–109.
[25] Y.J. Kang, Y. Feng, E.L. Hatcher, J. Cell. Physiol. 161 (1994) 589–596.
[26] M.T. Vincenzini, P. Marraccini, T. Iantomasi, F. Favilli, S. Pacini, M. Ruggiero, FEBS Lett. 320 (1993) 219–223.
[27] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[28] J.C. Fernandez-Checa, C. Ren, T.Y. Aw, M. Ookhtens, N. Kaplowitz, Am. J. Physiol. 255 (1988) G403–G408.
[29] M.T. Vincenzini, T. Iantomasi, F. Favilli, Biochim. Biophys. Acta 987 (1989) 29–37.
[30] M.T. Vincenzini, F. Favilli, T. Iantomasi, Biochim. Biophys. Acta 942 (1988) 107–114.
[31] C. Garcia-Ruiz, J.C. Fernandez-Checa, N. Kaplowitz, J. Biol. Chem. 267 (1992) 2256–22264.
[32] J.S. Cornell, A. Meister, Proc. Natl. Acad. Sci. USA 73 (1976) 420–422.
[33] M. Darbouy, M.N. Chobert, O. Lahuna, T. Okamoto, J.P. Bonvalet, N. Farman, Y. Laperche, Am. J. Physiol. 261 (1991) C1130–C1137.
[34] A. Blais, P. Bissonnette, A. Berteloot, J. Membr. Biol. 99 (1987) 113–125.
[35] T. Yada, S. Oiki, S. Ueda, Y. Okada, J. Membr. Biol. 112 (1989) 159–167.
[36] S. Lu, C. Garcia-Ruiz, J. Kuhlenkamp, M. Ookhtens, M. Salas-Prato, N. Kaplowitz, J. Biol. Chem. 265 (1990) 16088–16095.
[37] N. Ballatori, W.J. Dutczak, J. Biol. Chem. 269 (1994) 19731–19737.
[38] M. Inoue, Y. Morino, J. Biol. Chem. 260 (1985) 326–331.
[39] L.H. Lash, D.P. Jones, J. Biol. Chem. 259 (1984) 14508–14514.
[40] T.M. Hagen, D.P. Jones, Am. J. Physiol. 252 (1987) G607–G613.
[41] S. Lu, J.Y. Ge, H.Y. Huang, J. Kuhlenkamp, N. Kaplowitz, J. Clin. Invest. 92 (1993) 1188–1197.
[42] D.P. Jones, R.J. Coates, E.W. Flagg, J.W. Eley, G. Block, R.S. Greenberg, E.W. Gunter, B. Jackson, Nutr. Cancer 17 (1992) 57–75.
[43] L.J. Dahm, D.P. Jones, Toxicol. Appl. Pharmacol. 129 (1994) 272–282.