The Role of Glutathione in the Permeation Enhancing Effect of Thiolated Polymers

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Purpose. To verify or refute the mechanism of permeation enhancement with thiolated polymers via GSH by the use of NaFlu as marker for the paracellular permeation.

Methods. The capability of 0.5% polycarbophil cysteine conjugate (PCP-Cys) to reduce 0.02% oxidized glutathione (GSSG) was evaluated via iodometric titration in aqueous solution. Glutathione in its reduced form (GSH; 0.1%–0.4%) and in combination with 0.5% PCP-Cys were tested for their permeation enhancement of sodium fluorescein (NaFlu) and fluorescence labeled bacitracin (bac-FITC) used as paracellular markers. Permeation studies across guinea pig duodenum were carried out in Ussing-type chambers. Opening of the tight junctions was additionally monitored by transepithelial electrical resistance (TEER) measurements.

Results. PCP-Cys (0.5%) was shown to reduce 22.0% ± 8.2% of GSSG (0.02%) to GSH in aqueous solution at pH 7.0 and 37°C within 3 h. Permeation of NaFlu was shown to depend on the concentration of GSH. The apparent permeability coefficient (P_{app}) of NaFlu in buffer only was $4.98 \pm 0.5*10^{-6}$, while in the presence of 0.4% GSH a P_{app} of $9.31 \pm 0.92*10^{-6}$ was achieved, representing an enhancement ratio (R = P_{app} enhancer system/P_{app} control) of 1.86. The combination of GSH (0.4%) with PCP-Cys (0.5%) led to a significant ($p < 0.001$) improvement of R for NaFlu up to 2.93 accompanied by a decrease in TEER of 20.3% ± 1.4%. Incubation of bac-FITC with the same GSH / PCP-Cys combination led to an enhancement ratio of 2.06 within 3 h.

Conclusion. GSH plays an important role in the opening of tight junctions of intestinal epithelia. It would appear that PCP-Cys is able to reduce GSSG, prolonging the concentration of GSH at the apical membrane, resulting in significantly enhanced paracellular transport.

KEY WORDS: Ussing-type chamber; glutathione; small intestine; permeation enhancement.

INTRODUCTION

The oral bioavailability of many hydrophilic drugs such as heparins or peptide and protein drugs is often strongly limited by insufficient paracellular absorption from the GI tract. This absorption barrier is represented by epithelial cell membranes, interconnected by proteinaceous tight junctions (1). The knowledge of the mechanism that opens the tight junctions makes it possible to develop auxiliary agents increasing the paracellular flux. So far a large number of permeation enhancers has been identified (2). High molecular weight polymers such as chitosan and polyacrylates have received lots of attention (3). They display some additional advantages in comparison to low molecular weight enhancers, like mucoadhesive properties that allow them to remain concentrated at the area of drug absorption (3). As they will not be absorbed from the gut due to their high molecular weight (4), systemic side effects can be excluded. To optimize the properties of such polymers chemical modifications seem to be appropriate.

Based on this idea, a new generation of mucoadhesive polymers has been introduced in pharmaceutical literature. Due to the immobilization of free sulfhydryl groups onto various polymers their permeation enhancing effect on hydrophilic compounds, such as sodium fluorescein (NaFlu) or fluorescence labeled bacitracin (bac-FITC), has been increased (5–7). In addition, thiolated polymers or so-called "thiomers" exhibit improved mucoadhesive properties (8). However, the underlying mechanism for the permeation enhancing effect of thiomers remained unclear. It has been postulated that the mechanism involves the inhibition of protein tyrosine phosphatase (PTP) (6). Glutathione in its reduced form (GSH), exhibiting a thiol group was shown to interact with PTP (9–11) and it is therefore likely that GSH plays an important role in the permeation enhancement of paracelluar absorbable therapeutics across the intestinal mucosa. Furthermore, glutathione is known to be present in its reduced (GSH) and oxidized (GSSG) form at the apical side of the intestinal mucosa (12). Thiomers expressing reactive thiol groups may be able to reduce GSSG thereby increasing the amount of GSH at the apical surface. Hence, an improved permeation might be based on a PCP-Cys mediated increase in GSH concentration. It was the objective of this study to verify or refute the theory concerning the mechanism of permeation enhancement with thiolated polymers via GSH by the use of NaFlu as marker for the paracellular permeation. To demonstrate the practical relevance of the findings within this study permeation studies were also carried out with the model peptide drug bacitracin.

MATERIALS AND METHODS

Materials

Acivicin; bacitracin; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC); fluoresceinisothiocyanate (FITC); glutathione oxidized form (GSSG); (GSH); L-cysteine hydrochloride hydrate (L-cysteine); N-(2-hydroxyethyl)piperazine-N′-(2-ethane-sulfonic acid) (HEPES) and sodium fluorescein (NaFlu) were all purchased from Sigma, St. Louis, Missouri; polycarbophil (PCP, mol. wt >700 kDa; Noveon AA1) was donated by BFGoodrich, Brecksville, Ohio.

Synthesis of PCP-Cys Conjugates

The PCP-cysteine conjugates were synthesized according to the method previously described by our research group

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ABBREVIATIONS: bac-FITC, fluorescence labeled bacitracin; EDAC, carbodiimide hydrochloride; FITC, fluoresceinisothiocyanate; GI, gastrointestinal tract; GSH, glutathione in its reduced form; GSSG, glutathione oxidized form; NaFlu, sodium fluorescein; PCP-Cys, polycarbophil cysteine conjugate; PTP, protein tyrosine phosphatase; TEER, transepithelial electrical resistance.

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(13). In brief, after 45 min of EDAC activation (50 mM) of one g neutralized PCP (NaPCP) hydrated in 250 ml of demineralized water, two g of L-cysteine were added. The pH was adjusted to 6 by adding 1 M NaOH during a 3 h reaction time at room temperature. The resulting conjugate was isolated by dialyzing at 10°C in the dark against 1 mM HCl containing 2 μM EDTA, two-times against the same medium but containing 1% NaCl and finally exhaustively against 0.5 mM HCl. The pH of the dialyzed polymer-cysteine conjugate was adjusted to 4.5 with 1 M NaOH. Thereafter the conjugate was lyophilized by drying the frozen aqueous polymer solutions at –30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany). Control polymer was prepared and isolated in the same way as the PCP-Cys conjugate but EDAC was omitted during the coupling reaction. The polymer-cysteine conjugate was stored at 4°C until use.

Determination of the Thiol Group Content

The amount of modification was determined by quantifying the amount of covalently attached thiol moieties via iodometric titration at pH 3.0 (1 mM iodine; indicator: starch) (14).

FITC Labeling of Bacitracin

FITC was conjugated to the peptide drug according to the method previously described by our research group (6). In brief, 2 mg of FITC dissolved in 1 ml of dimethylsulfoxide was slowly added in aliquot volumes of 25 μl to 40 mg of bacitracin dissolved in 20 ml of 0.1 M Na_2CO_3. After 8 h of incubation at 4°C, the coupling reaction was stopped by the addition of NH_4Cl, to give a final concentration of 50 mM. The resulting bacitracin-FITC (bac-FITC) conjugate was incubated for 2 h at 4°C, isolated by gel filtration (Sephadex G15, Pharmacia Uppsala, Sweden) and lyophilized as described above. The amount of covalently attached FITC on the peptide drug was determined by measuring the absorbance at 495 nm of 1 mg drug dissolved in 1 ml of demineralized water at pH of 7.4, using a UV/Vis spectrophotometer (Lambda 16, Perkin Elmer; Vienna, Austria). A standard curve of FITC was prepared in demineralized water at pH 7.4.

Permeation Studies

According to previous studies Ussing-type chambers with a surface area of 0.64 cm^2 were used to carry out permeation studies with NaFlu and bac-FITC (6). The pH of the freshly prepared incubation medium containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 40 mM HEPES was adjusted to 7.0. Immediately after sacrificing the animal the first 10 cm of the small intestine (duodenum) of the guinea pig was excised and mounted in the Ussing-type chamber, without stripping off the underlying muscle layer. All experiments were preformed in triplicate in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. After 15–20 min of preincubation the solution in the donor chamber was substituted by the same medium but containing either GSH in different concentrations or 0.4% (w/v) GSSG. Sodium fluorescein 0.001% (w/v) was additionally added as paracellular marker. Further studies were carried out by substituting with a combination of 0.5% (w/v) PCP-Cys with either 0.4% GSH or 0.4% GSSG in incubation medium containing 0.001% NaFlu. To evaluate the effect of gamma glutamyl transferase (gamma-GT) leading to the hydrolization of glutathione, the same experiment was carried out in the presence of 0.5 M acivicin, an inhibitor of gamma-GT. The effect of 0.5% PCP-Cys in combination with 0.4% GSH in a 0.1% bac-FITC incubation medium was also tested. Control studies were carried out with NaFlu or bac-FITC in incubation medium. Over a 3 h incubation period 100 μL samples were taken from the acceptor chamber every 30 min and the volume was replaced by incubation medium equilibrated at 37°C. The permeation of NaFlu or bac-FITC was evaluated by measuring the amount of permeated test compound in the acceptor chamber using a fluorimeter (SLT; Spectra Fluor; Tecan, Austria). Cumulative corrections were made for the prior removed samples in determining the total amount permeated.

Viability Studies

After permeation studies the medium was removed from the donor chamber and 1 ml trypan blue dye was added and the mucosa was incubated for 30 min. Microscopic investigations demonstrated, that the mucus was still present and that the viability of the intestinal membrane was guaranteed as no blue color was detectable within the cells. These observations were in good agreement with the results of viability studies previously published by various research groups e.g., (6,15).

Measurement of the Transepithelial Electrical Resistance (TEER)

A Millicell® ERS meter (Millipore Corp., Bedford, MA) connected to a pair of side-by-side electrodes was used to monitor the effect on the TEER of the intestinal mucosa. Measurements were performed every 5 min before applying the test compounds and then every 30 min within 3 h.

Reducing Activity of PCP-Cys on GSSG

Polymer-cysteine conjugate (50 mg) was hydrated in 7 ml of aqueous 1.8% NaCl. After the addition of 1 ml of 0.02% (w/v) GSSG, the pH was adjusted to 7.0 with 1 M NaOH and the volume was made up to 10 ml with aqueous 1.8% NaCl. The reaction mixture was incubated at 37°C while shaking permanently. Samples of 1 ml were withdrawn at predetermined time points and centrifuged for 3 min at 17,000 g. Afterwards 100 μL of the supernatant containing GSH and GSSG were collected. The amount of GSH was detected by iodometric titration of the thiol group of reduced glutathione. Control samples were prepared as described above with unmodified polymer or by omitting the thiolated polymer. The amount of reduced glutathione was calculated from a simultaneously run standard curve, which was obtained from samples containing GSH in increasing concentrations.

Determination of Autoxidation of GSH

For autoxidation studies 2.5 mg of GSH was incubated in 10 ml of a 100 mM phosphate buffer pH 7.4. The solution was incubated in an incubator (Memmert GmbH, Modell 500, Germany) at 37°C. Samples of 200 μL were withdrawn at predetermined time points. The amount of remaining GSH was detected spectrophotometrically using Ellman's reagent.

Data Analyses

Apparent permeability coefficients (P_{app}) for NaFlu and bac-FITC were calculated according to the following equation:

$$P_{app} = Q/A*c*t \quad (1)$$

where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount permeated throughout the incubation time (μg), A is the diffusion area of the Ussing-type chamber (cm^2), c is the initial concentration of the marker in the donor compartment ($\mu g/cm^3$), and t is the total time of the experiment (s).

Transport enhancement ratios (R) were calculated from P_{app} values by:

$$R = P_{app}\,(\text{sample})/P_{app}\,(\text{control}) \quad (2)$$

Statistical data analyses were performed using the student *t* test with $p < 0.05$ as the minimal level of significance.

RESULTS

Characterization of the Polymer-Cysteine Conjugate

Former studies demonstrated that PCP-Cys conjugates within the range of 180–344 μM sulfhydryl groups per gram polymer exhibit the strongest permeation enhancing properties (6). To synthesize a PCP-Cys conjugate exhibiting a similar coupling rate, the same reaction conditions as used previously were chosen. The resulting PCP-Cys conjugate displayed 258.5 ± 20.1 μM sulfhydryl groups per gram polymer, underlying the reproducibility of this synthetic pathway. Due to this conjugation a degree of substitution with average 0.025 ± 0.007 cysteine units per acrylic acid subunit could be achieved. Omitting the coupling reagent EDAC during the reaction led to a polymer exhibiting a negligible amount of sulfhydryl groups. The stability, swelling behavior, and mucoadhesive properties of various polycarbophil-cysteine conjugates have already been evaluated (13,14). The features of the thiolated polymer used herein were in good agreement with the already characterized PCP-Cys conjugates.

Determination of the Permeation Enhancing Effect of GSH and GSSG

To determine the permeation enhancing effect of GSH, the transport of NaFlu was evaluated *in vitro* across guinea pig mucosa in presence of various concentrations of GSH. It was demonstrated that increasing the amount of GSH from 0.1% up to 0.4% leads to significantly improved permeation ratios of NaFlu (Fig. 1. , means ±S.D. n = 3; $p < 0.05$). A maximum P_{app} of 9.31 ± 0.92 $*10^{-6}$ was achieved, which represents an enhancement ratio of 1.86. The enhancement results are depicted in Table I. For the control, incubation medium without polymer, there was no significant change in the TEER detectable, whereas a maximum decrease of 15.12% ± 0.36% was observed for 0.4% GSH indicating the opening of the tight junctions. This result underlines the potential of GSH as permeation enhancer for hydrophilic drugs.

In contrast to reduced glutathione its oxidized form showed only a weak permeation enhancing effect. Even the highest tested concentration of GSSG (0.4%) led to an enhancement ratio of merely 1.31. This permeation enhancing effect of GSSG at all can be explained by the reducing activity of glutathione reductase being present in intestinal mucosa generating GSH (12).

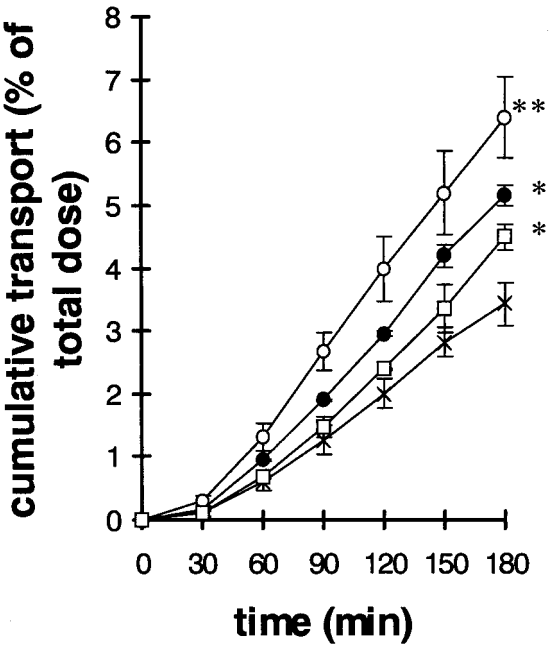

Fig. 1. Transport of NaFlu across small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of NaFlu applied to the luminal side of the mucosa. Control without glutathione (X); 0.1% (w/v) GSH (□); 0.2% (w/v) GSH (●); 0.4% (w/v) GSH (○); (means ± S.D.; n = 3). *, differs from control without GSH, $p < 0.01$; **, differs from control without GSH, $p < 0.001$.

Reduction of GSSG by the use of PCP-Cys

In order to evaluate the potential of PCP-Cys to reduce GSSG to GSH, the polymer was incubated with GSSG as described above. On average 22.0% ± 8.2% of GSSG was reduced to GSH within 180 min of incubation (Fig. 2) . The underlying mechanism for the reduction of GSSG by PCP-Cys is shown in Fig. 3. The cysteine groups of the polymer exhibit a pKa of 8.35, calculated by the ACD-Software (Toronto, Canada). According to the following equation (16): $f_{ion} = 100/[1 + 10^{(pH + pKa)}]$, 4.27% of the cysteine moieties will have reactive thiolate anions ($-S^-$) at pH 7.0. On the other hand GSH with a calculated pKa of 9.25, will only have 0.55% of $-S^-$ and will therefore play a minor role in the reducing mechanism. This is according to Dahm and Jones's findings, which suggest that cysteine could function in the reduction of GSSG in the lumen (17). Control experiments of GSSG incubated with unmodified polymer or without polymer showed no detectable thiol groups within the incubation period.

Infuence of PCP-Cys in Combination with GSH or GSSG on the Permeation of Naflu and Bac-FITC

The permeation enhancing effect of 0.4% GSSG in combination with 0.5% PCP-Cys on the transport of NaFlu was evaluated *in vitro* with small intestinal tissue isolated from guinea pig. The combination led to a significantly improved permeation of the model drug (Fig. 4) accompanied by a decrease in TEER by 18.7% ± 1.5%. The enhancement ratio

Table I. Comparison of the Influence of GSH; GSSG with or without PCP-Cys on the Apparent Permeability Coefficient (P_{app}) for Sodium Fluorescein and Bacitracin-FITC across the Intestinal Mucosa of Guinea Pigs

Paracellular marker	Test compound	Apparent permeability coefficient [$P_{app}*10^{-6}$ (cm/sec)], means ± S.D., n = 3	Enhancement ratio (P_{app} enhancer system/P_{app} control)	Reduction of TEER value
Sodium	control (buffer only)	4.98 ± 0.50	1	
fluorescein	0.1% GSH	6.54 ± 0.30	1.31	5.2% ± 1.0%
	0.2% GSH	7.51 ± 0.24	1.50	8.1% ± 1.3%
	0.4% GSH	9.31 ± 0.92	1.86	15.1% ± 0.4%
	0.4% GSSG	6.55 ± 0.07	1.31	4.8% ± 0.7%
	0.4% GSSG/0.5% PCP-Cys	10.16 ± 1.64	2.04	18.7% ± 1.5%
	0.4% GSH/0.5% PCP-Cys	14.64 ± 0.93	2.93	20.3% ± 1.4%
bac-FITC	control (buffer only)	4.81 ± 0.64	1	
	0.4% GSH/0.5% PCP-Cys	9.94 ± 0.82	2.06	19.8% ± 1.2%

was significantly increased to 2.04 (Table I). To determine the effect of GSH in combination with PCP-Cys the same experiment was repeated but 0.4% GSH was used instead of GSSG. A significant increase of the enhancement ratio of 2.93 was monitored as depicted in Fig. 5 and the TEER decreased by 20.3% ± 1.4%. Concentrations of GSH above 0.4% in combination with the thiomer did not lead to higher permeation of NaFlu. The enhancing effect of GSH in combination with PCP-Cys was also tested on bac-FITC, a peptide drug with a molecular weight of approximately 1.4 kDa. Chemical interactions of the peptide with the polymer can generally be excluded, because it bears no thiol moieties being able to form disulfide bonds. On average, approximately 0.04 mole FITC were bound to one mole bacitracin. Within this experiment even for the higher molecular mass compound a significant increase in the permeation was detected (Fig. 6). A maximum P_{app} of 9.94 ± 0.82 $*10^{-6}$ was reached, which represents an enhancement ratio of 2.06 (Table I). These are much higher permeation ratios of the model compound as already demonstrated in former studies (6).

In order to demonstrate that the amount of active GSH will not be changed via the gamma-GT, permeation studies with PCP-Cys in combination with GSH and acivicin, a selective gamma-GT inhibitor, were carried out. This experiment showed no significant difference in the permeation of Na-Flu across small intestinal mucosa of guinea pigs during the incubation period (data not shown).

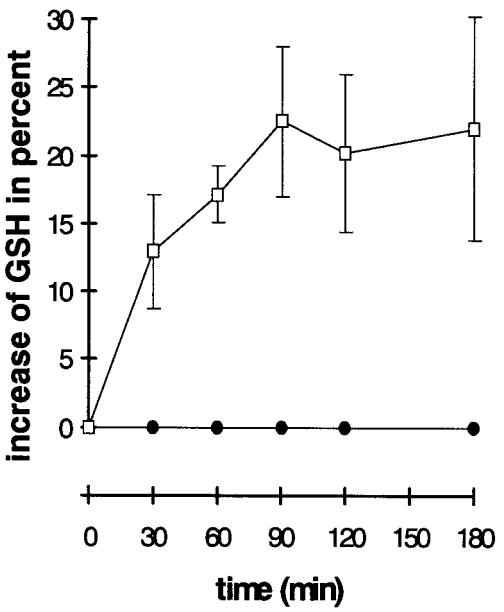

Fig. 2. Incubation of 0.02% (w/v) oxidized glutathione (GSSG) with 0.5% (w/v) PCP-Cys (□) and with unmodified PCP (●) at 37°C pH 7.0. Indicated values of comprised GSH in percent are means ± SD of at least three experiments.

Autoxidation of GSH

As reduced glutathione was shown to be the active form for permeation enhancement, it is important to demonstrate its behavior at physiological conditions. Therefore, 0.25% of GSH was incubated for 24 h at 37°C and pH 7.4. Within a incubation period of 3 h 83.6% ± 3.2% of the used GSH were

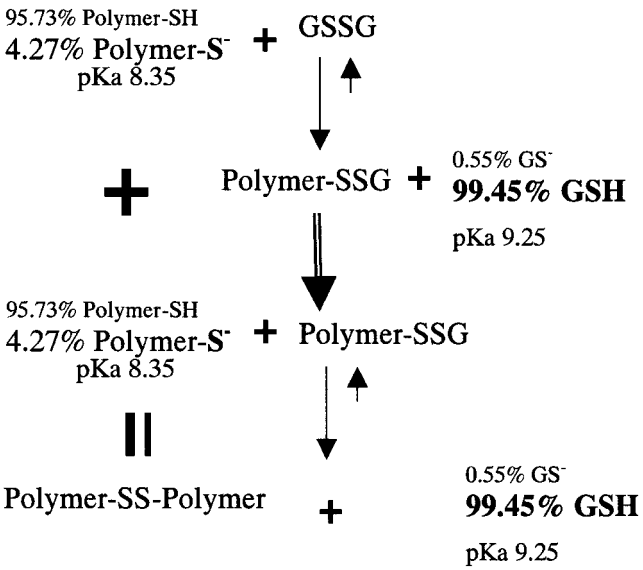

Fig. 3. Proposed reaction scheme for reduction of the oxidized form of glutathione via PCP-Cys at pH 7.0.

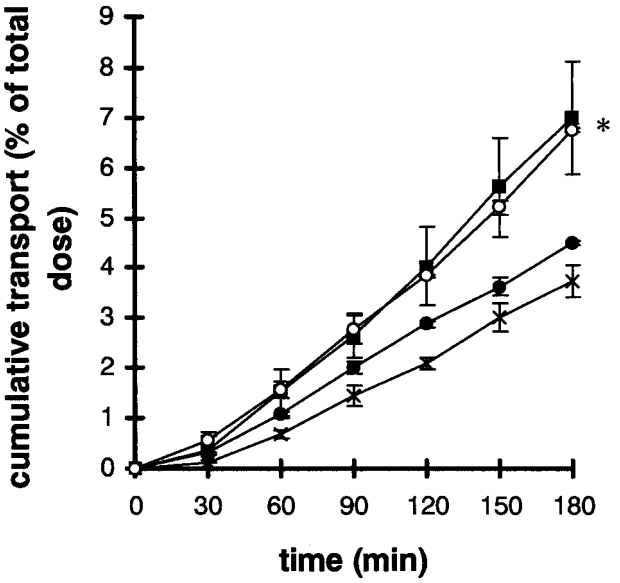

Fig. 4. Transport of NaFlu across small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of NaFlu applied to the luminal side of the mucosa. Control without enhancer **(X)**; 0.5% (w/v) PCP-Cys (○); 0.4% (w/v) GSSG (●); 0.4% (w/v) GSSG and 0.5% (w/v) PCP-Cys (■); (means ± S.D.; n = 3). *, both differ from 0.4% GSSG, $p < 0.001$.

still remaining which was important for the permeation studies. Ongoing studies over a time period of 24 h demonstrated a residual amount of GSH of only 10.1% ± 2.5%.

DISCUSSION

Our present study demonstrated that GSH plays an important role in the opening of the tight junctions for improved paracellular drug transport. Focusing on the compounds involved in the opening and closing mechanism of tight junctions the membrane protein occludin is believed to play a crucial role (18). This protein expresses an intracellular region and two extracellular loops reaching into the paracellular space (19). They express numerous tyrosine residues, which can be phosphorylated leading to increased tight junctional permeability (18). The enzyme protein tyrosine phosphatase (PTP) is able to dephosphorylate these groups, which results in closing of the tight junctions (18). Consequently, inhibition of PTP must lead to an improved permeability. As PTP shows a cysteine moiety as active site being responsible for its activity, GSH was shown to be able to inhibit PTP activity via a disulfide bond formation within 5 min. by approximately 100% (9). Taking into consideration that GSH has high activity for PTP inhibition a permeation enhancing effect of GSH could be expected. Until now it has been demonstrated that GSH is involved in a H_2O_2 mediated increased paracellular permeability in Caco-2 cell monolayers (11). The results of permeation studies in Ussing type chambers with increasing amounts of GSH substantiated the inhibition theory of PTP as indicated in Figure 1. Amounts of GSH up to 0.4% resulted in a higher enhancing effect accompanied by a decrease in TEER. Additional studies with GSSG showed a comparatively much lower permeation enhancement. These results point out that for PTP inhibition the free thiol group is essential. Due to an autoxidation of GSH and its oxidation catalyzed on the cell surface (20), the amount of active GSH decreases thereby leading to lower concentrations resulting in a decreased permeation enhancement. To compensate the loss of GSH due to the formation of GSSG, a chemical reduction of GSSG would be favorable. A small amount of

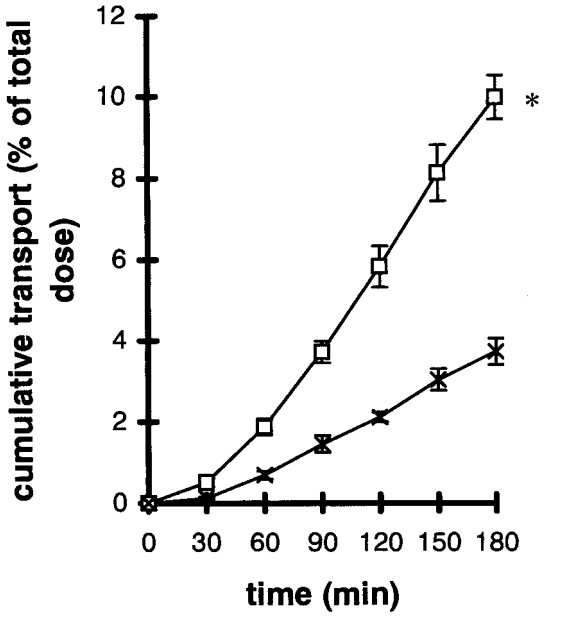

Fig. 5. Transport of NaFlu across small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of NaFlu applied to the luminal side of the mucosa. Control without enhancer **(X)**; 0.4% (w/v) GSH and 0.5% (w/v) PCP-Cys (□); (means ± S.D.; n = 3). *, differs from control, $p < 0.001$.

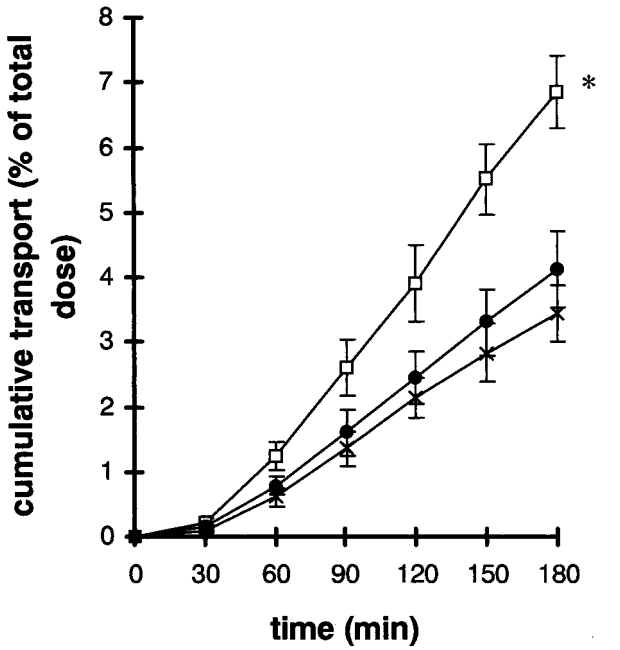

Fig. 6. Transport of bac-FITC across small intestinal mucosa of guinea pigs. Transport data are expressed as percentage of the total dose of bac-FITC applied to the luminal side of the mucosa. Control without enhancer **(X)**; 0.5% (w/v) PCP-Cys (●); 0.4% (w/v) GSH and 0.5% (w/v) PCP-Cys (□); (means ± S.D.; n = 3). *, differs from control, $p < 0.001$.

GSSG is known to be reduced to GSH inside the mucosa by GSSG reductase (12) explaining the weak permeation enhancing effect which was achieved by the addition of GSSG (Fig. 4). According to the results shown herein PCP-Cys is able to reduce GSSG, which will further strongly increase the amount of GSH. The reducing process as depicted in Fig. 3 can be explained by the pKa of PCP-Cys and GSH. As the pKa of the polymer attached thiol groups is 8.35, they are in their active ionized form by 4.27% at pH 7.0 and a nucleophilic attack on GSSG takes place quite rapidly. In contrast, the thiol group of GSH is only to a negligible extent involved in the nucleophilic attack of disulfide bonds, as the pKa of the thiol moiety is 9.25. Only 0.55% of the thiol groups will be active, which is an approximately 8-fold lower activity than for PCP-Cys. GSH remains therefore stable in its reduced form. Higher pH at the absorption area should thereby increase the activity of PCP-Cys as there will be more active thiol groups available. This leads to higher GSH concentrations followed by an improved opening of the tight junctions. PCP-Cys, known to express a high buffer capability (21), can be adjusted to a higher pH, which can then be guaranteed for a long time period at the absorption area.

Former permeation studies demonstrated an enhancing effect of PCP-Cys suggested to be referred to a PTP-inhibition (6). This theory can now be verified by the mechanism as shown in Figure 7, substantiated by the already discussed results. Furthermore, no permeation enhancing effect was observed by addition of free cysteine-HCl. As cysteine is absorbed very fast by the cells (23), no interaction with PTP and/or GSSG can occur. GSH on the other hand will be absorbed much slower thereby remaining concentrated on the membrane for a longer time period (23). Hence, only GSH and not cysteine leads to the enhancing effect. According to this consideration, the covalent attachment of cysteine onto the polymer is substantial. A dilution effect of the attached cysteine moieties can be excluded due to the mucoadhesive and cohesive properties of this polymer (8). It results in higher concentrations of reactive thiol moieties at the permeation area producing GSH by the reduction of GSSG. The concentration of GSH on the absorption membrane is raised by PCP-Cys. GSH provides permeation enhancement and the PCP-Cys conjugate reduces the oxidized form of glutathione that is generated on the cell surface. Permeation enhancement ratios went to 2.93, this was the highest ratio within Ussing-type chamber systems with small intestinal tissue for NaFlu. Additionally, systemic side effects for PCP-Cys can be generally excluded, as it is not absorbed from the small intestine (4). The change in the concentration of GSH, being secreted from small intestinal cells, should not lead to side effects as well.

CONCLUSION

Due to its reducing activity on GSSG PCP-Cys is able to increase the concentration of GSH on the absorption membrane. GSH in turn is able to inhibit protein tyrosine phosphatase via disulfide bond formation with its active site cysteine resulting in the disruption of tight junction integrity. This leads consequently to an enhanced permeation of hydrophilic compounds such as peptides. The knowledge of the mechanism might provide a platform for further improvement of the permeation enhancing effect of e.g. matrix tablet systems or micro- and nanoparticles based on thiomers.

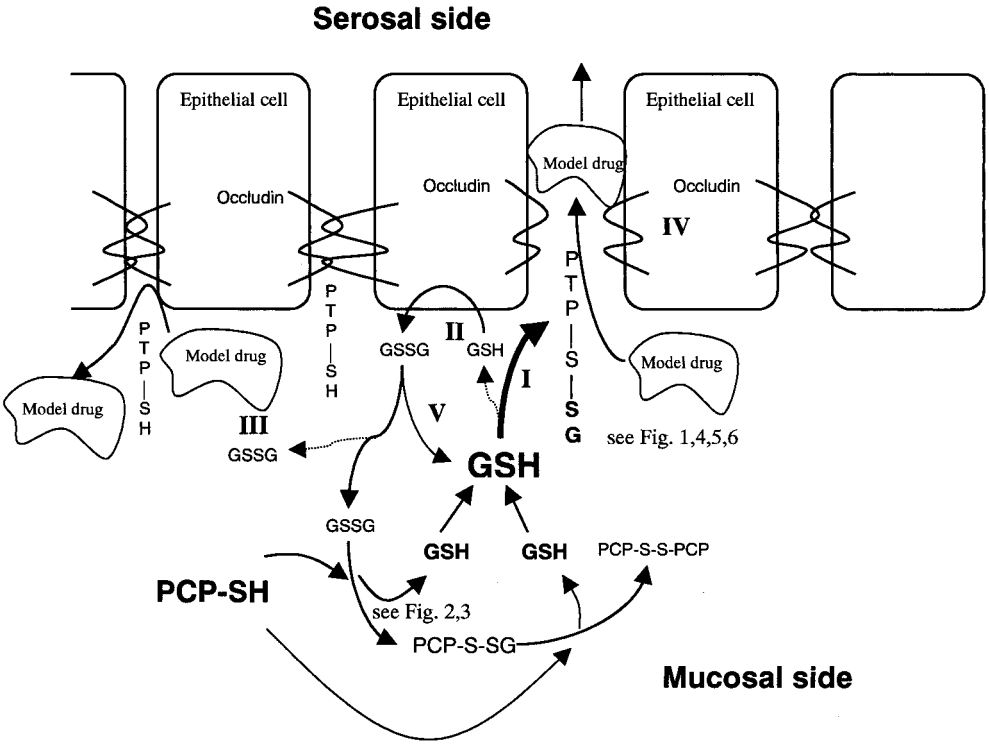

Fig. 7. Proposed mechanism of GSH induced opening of the tight junctions via inhibition of PTP. (**I**) Inactivation of PTP via covalent attachment of GSH on the active site Cysteine 215: (9); (**II**) Oxidation of GSH to GSSG catalyzed by the cells: (20); (**III**) Metabolism of GSSG via gamma-Glutamyltransferase: (20); (**IV**) Increased tight junction permeability by the inhibition of PTP via a specific tyrosine phosphatase inhibitor: (22); (**V**) Reduction of GSSG in the gastrointestinal mucosa by GSSG reductase: (12).

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REFERENCES

1. S. Tsukita, M. Furuse, and M. Itoh. Multifunctional strands in tight junctions. *Nature Re. Mol. Cell Biol.* **2**:285–293 (2001).
2. B. J. Aungst. Intestinal permeation enhancers. *J. Pharm. Sci.* **89**: 429–442 (2000).
3. H. L. Lueßen, C. O. Rentel, A. F. Kotzé, C.-M. Lehr, A. G. deBoer, J. C. Verhoef, and H. E. Junginger. Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro. *J. Control. Release* **45**:15–23 (1997).
4. Y. Takehisa, S. Takaharu, T. Eiji, and N. Osamu. Disposition of polycarbophil calcium (HSR-237) in rats and dogs. *Iyakuhin Kenkyu* **28**:23–32 (1997).
5. A. E. Clausen and A. Bernkop-Schnürch. Thiolated carboxymethylcellulose: in vitro evaluation of its permeation enhancing effect on peptide drugs. *Eur. J. Pharm. Biopharm.* **51**:25–32 (2001).
6. A. E. Clausen and A. Bernkop-Schnürch. In vitro evaluation of the permeation-enhancing effect of thiolated polycarbophil. *J. Pharm. Sci.* **89**:1253–1261 (2000).
7. A. Bernkop-Schnürch, U. M. Brandt, and A. E. Clausen. Synthesis and in vitro Evaluation of Chitosan-Cysteine Conjugates. *Sci. Pharm.* **67**:196–208 (1999).
8. A. Bernkop-Schnürch, V. Schwarz, and S. Steininger. Polymers with thiol groups: A new generation of mucoadhesive polymers? *Pharm. Res.* **16**:876–881 (1999).
9. W. C. Barrett, J. P. DeGnore, S. Konig, H. M. Fales, Y. F. Keng, Z. Y. Zhang, M. B. Yim, and P. B. Chock. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* **38**:6699–6705 (1999).
10. D. Degl'Innocenti, A. Caselli, F. Rosati, R. Marzocchini, G. Manao, G. Camici, and G. Ramponi. Thiolation of low-Mr phosphotyrosine protein phosphatase by thiol-disulfides. *Life* **48**:505–511 (1999).
11. R. K. Rao, L. Li, R. D. Baker, S. S. Baker, and A. Gupta. Glutathione oxidation and PTPase inhibition by hydrogen peroxide in Caco-2 cell monolayer. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**:G332–G340 (2000).
12. C. P. Siegers, D. Riemann, E. Thies, and M. Younes. Glutathione and GSH-dependent enzymes in the gastrointestinal mucosa of the rat. *Cancer Lett.* **40**:71–76 (1988).
13. A. Bernkop-Schnürch and S. Steininger. Synthesis and characterisation of mucoadhesive thiolated polymers. *Int. J. Pharm.* **194**: 239–247 (2000).
14. A. Bernkop-Schnürch, S. Scholler, and R. G. Biebel. Development of controlled drug release systems based on thiolated polymers. *J. Control. Release* **66**:39–48 (2000).
15. T. Uchiyama, A. Kotani, T. Kishida, H. Tatsumi, A. Okamoto, T. Fujita, M. Murakami, S. Muranishi, and A. Yamamoto. Effects of various protease inhibitors on the stability and permeability of [D-Ala2, D-Leu5]enkephalin in the rat intestine: Comparison with leucine enkephalin. *J. Pharm. Sci.* **87**:448–452 (1998).
16. A. Martin, J. Swarbrick, and A. Cammarata. *Physical Pharmacy* 3rd edition, Lea and Febriger, Philadelphia, Pennsylvania 1983.
17. L. J. Dahm and D. P. Jones. Rat jejunum controls luminal thiol-disulfide redox. *J. Nutr.* **130**:2739–2745 (2000).
18. C. B. Collares-Buzato, M. A. Jepson, N. L. Simmons, and B. H. Hirst. Increased tyrosine phosphorylation causes redistribution of adherens junction and tight junction proteins and perturbs paracellular barrier function in MDCK epithelia. *Eur. J. Cell Biol.* **76**:85–92 (1998).
19. M. Furuse, T. Hirae, M. Itoh, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions. *J. Cell Biol.* **6**:1777–1788 (1993).
20. R. Grafstrom, A. H. Stead, and S. Orrenius. Metabolism of extracellular glutathione in rat small-intestinal mucosa. *Eur. J. Biochem.* **106**:571–577 (1980).
21. A. Bernkop-Schnürch and B. Gilge. Anionic mucoadhesive polymers as auxiliary agents for the peroral administration of (poly) peptide drugs: influence of the gastric juice. *Drug Dev. Ind. Pharm.* **26**:107–113 (2000).
22. J. M. Staddon, K. Herrenknecht, S. Smales, and L. L. Rubin. Evidence that tyrosine phosphorylation may increase tight junction permeability. *J. Cell Sci.* **108**:609–619 (1995).
23. K. Yoshimura, Y. Iwauchi, S. Sugiyama, T. Kuwamura, Y. Odaka, T. Satoh, and H. Kitagawa. Transport of L-cysteine an dreduced glutathione through biologic membranes. *Res. Commun. Chem. Pathol. Pharmacol.* **37**:171–186 (1982).