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Investigation of the Transport of Intact Glutathione in Human and Rat Type II Pneumocytes

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The aim of the study was to investigate whether there is transmembrane transport of intact glutathione ([^{3}H]-GSH, 0.1 μCi) in rat and human type II pneumocytes (T2P), and if this transport might be dependent on the redox state of the extracellular fluid. The T2P were pretreated with acivicin (250 μM) to inhibit γ-glutamyl-transferase activity and with L-buthionine-[SR]-sulfoximine (1 mM) to inhibit intracellular GSH synthesis. After 48 h in culture, initial GSH influx rate was 0.70 ± 0.20 nmol/min/mg protein (37°C) and 0.35 ± 0.04 nmol/min/mg protein (4°C) during the first 5 min in rat T2P. In human T2P, the initial GSH influx rate was 0.36 ± 0.30 nmol/min/mg protein (37°C) and 0.32 ± 0.06 nmol/min/mg protein (4°C) during the first 10 min. Thereafter no further influx was found. The influx of 1 mM GSH in freshly isolated rat and human T2P in suspension was 2.3 ± 0.3 and 1.2 ± 0.3 nmol/mg protein after 15 min at 37°C, and 2.8 ± 0.2 and 1.0 ± 0.3 nmol/mg protein at 4°C, respectively. When GSH influx was studied at different concentrations between 0 and 40 mM, a linear increase without saturation or difference between 37°C and 4°C was found. Pre-exposure to ouabain had no effect on GSH influx. Efflux of GSH was stimulated and influx inhibited by pre-exposure of the cells to reduced thiols, while disulphides inhibited efflux and favoured inward uptake. Thus, in human and rat T2P a GSH-carrier exists which operates as an effluxer. At GSH concentrations in the physiological range no uptake is seen, but some uptake can be observed at GSH concentrations above normal physiological levels. The uptake appears to be energy-independent and non-saturable. Efflux of GSH is stimulated and influx inhibited by reduced thiols, while disulphides inhibit the efflux and favour inward uptake. GSH uptake in T2P thus may depend on concentration gradients and driving forces, such as the redox state of the extracellular fluid.

Keywords: Reduced thiols; disulphides; transport mechanism; cystine; dithiothreitol; cell culture

Abbreviations: BSO, L-buthionine-[SR]-sulfoximine; BSA, bovine serum albumin; γ-GT, γ-glutamyltransferase; GSH, glutathione; GSSG, oxidized GSH; DTT, dithiothreitol; NAC, N-acetylcysteine; DTNB, 5,5-dithio-(2-nitro)-benzoic acid; FCS, fetal calf serum; SSA, 5-sulfosalicylic acid; PBS^+ and PBS^-, phosphate buffered saline with or without calcium and magnesium, respectively; V_{max}, the maximal rate of uptake; K_m, the medium concentration at which the rate of uptake is half V_{max}; dpm, disintegrations per minute; cpm, counts per minute

INTRODUCTION

Glutathione (GSH) is the major antioxidant in mammalian tissues.[1–3] The level of intracellular GSH is the final outcome of a complex process,

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which includes the uptake of constituents of the GSH molecule, intracellular GSH synthesis, and the efflux of intact GSH and GSSG.[4] Studies in liver cells have demonstrated that the efflux of GSH is determined by the extracellular redox state.[5–7] In a previous study we have found indirect evidence that this mechanism might also occur in alveolar type II cells: *in vivo* treatment of rats with 200 mg/kg N-acetylcysteine (NAC) i.p, for 10 days unexpectedly decreased intracellular GSH levels in isolated type II cells in parallel with increased GSH levels in the epithelial lining fluid.[8] In addition to the well-known uptake of the amino acid constituents of GSH, it has been concluded, mainly on the basis of work from one research group, that intact GSH is taken up via an energy- and sodium-dependent pathway in cells of epithelial origin, such as intestinal cells, isolated kidney cells, and also rat alveolar type II cells.[9–14] So far, it is unknown what the kinetic properties of this system are, and if such an uptake pathway for intact GSH also exists in human type II cells.

The aim of our study was to study GSH transport in rat and human type II pneumocytes, to investigate if the transport system in type II cells is bidirectional, and whether release or uptake of GSH is influenced by the concentration gradient and redox state of the extracellular fluid.

MATERIAL AND METHODS

Animals and Materials

Male Wistar rats (140–170 g) were obtained from an in-house strain. [2-^{3}H-glycine] glutathione [14.6 Ci/mmol (1 μCi/μl)] was purchased from Isobio Ltd. (Fleurus, Belgium); emulsifier safe scintillant and plastic scintillation vials (5 ml) were purchased from Packard N.V. (Zellik, Belgium). Acivicin [L-(αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid], dithiothreitol (DTT), L-buthionine-[SR]-sulfoximine (BSO), trypsin type I (EC 3.4.214), trizma base (Tris), bovine serum albumin (BSA), DNAse I, glutathione reductase type IV (EC 1.6.4.2, 100 U/ml), β-nicotinamide-adenine-dinucleotide phosphate, reduced form (β-NADPH), glutathione (GSH), oxidized glutathione (GSSG), penicillamine, cystine, N-acetylcysteine (NAC), xanthine, xanthine oxidase (EC 1.1.3.22) and percoll were all purchased from Sigma Germany (Filter Service NV/SA, Eupen, Belgium). Waymouth's 752/1 medium, fungizone (amphotericin 250 μg/ml), L-glutamine (200 mM), penicillin–streptomycin solution (10,000 U–10,000 μg/ml respectively), Hepes, and fetal calf serum (FCS) were purchased from Gibco (Merelbeke, Belgium). Protein assay dye solution was purchased from Bio-Rad (Brussels, Belgium). All other chemicals were purchased from U.C.B. Belgium (Vel NV/SA, Leuven, Belgium). Ninety-six-well cell-culture plates coated with extracellular matrix (ECM) were purchased from Biological Industries, Glasgow, U.K. PBS^- is phosphate-buffered saline (130 mM NaCl, 5.2 mM KCl, 10.6 mM Hepes and 2.6 mM Na_2HPO_4, pH 7.4), PBS^+ is the same buffer with the addition of $CaCl_2$ (1.9 mM) and $MgCl_2$ (1.3 mM).

Isolation of Human and Rat Type II Pneumocytes

Isolation of human type II cells was according to Hoet *et al.*[15] The cells were used 48 h after plating and were first rinsed with PBS^+ to remove non-adherent cells. This procedure yielded more than 90% viable type II pneumocytes, as determined by trypan blue exclusion and alkaline phosphatase staining. After 48 h, purity had increased to 95% after washing of the cells three times with PBS^+. Rat type II cell isolation was also done according to Hoet *et al.*[15] as adapted from Richards *et al.*[16]

GSH Influx Studies in Rat and Human Type II Cells

Two days after isolation, at which time the cells had reached almost confluent monolayers (more than 95% coverage of the well surface), the cells

were washed three times with PBS^+, and then pre-exposed to 250 μM acivicin, which inhibits γ-GT activity by >99%,[17] and to 1 mM BSO for 30 min, which inhibits GSH synthesis by more than 90%.[18] This was followed by several experiments:

(a) Exposure to 1 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 0, 5, 10, 15, or 30 min.
(b) Exposure to 1 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 0, 5, 10, 15, or 30 min after an additional pre-exposure of the cells to 2 mM DTT or 1 mM cystine for 1 h.
(c) Exposure to 250 μM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 0, 1, 2, 3, 4, 6, 8,10 min.
(d) Exposure to 0.5, 1, 2.5, 5, 10, 20 and 40 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ adjusted for pH for 15 min.
(e) To explain the discrepancy between our results and those of other investigators,[9–14] we tried to follow their protocol as closely as possible and studied, therefore, the effect of the incubation medium (PBS^+ vs. Krebs–Henseleit) and the effect of pre-equilibration of the medium with 95% O_2 – 5% CO_2 (100% N_2 was used as a control) on the uptake of 1 mM [^{3}H]-GSH (0.1 μCi for 5 min). To study the possibility that not GSH but GSSG had been taken up in the studies presented by others,[9–14] [^{3}H]-GSH (0.1 μCi) was pre-exposed to 0.5 mM xanthine plus 10 μl xanthine oxidase (25 U/1.6 ml) for 30 min to oxidize the GSH.
(f) Exposure to 1 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 15 min with and without 1 mM ouabain.

The specific activity of [^{3}H]-GSH used here was 0.5 μCi/μmol GSH, which is five times higher than the specific activity used by others.[9–14]

Type II cells were also cultured on 25 mm tissue culture inserts (pore size 0.2 μm) purchased from Nunc for 48 h (0.5×10^6 cells per well), so that a monolayer was formed. Subsequently, the cells were pre-exposed to 250 μM acivicin and 1 mM BSO for 30 min, followed by exposure to 1 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 0, 5, 10, 15, 20, or 30 min either via the upper or the lower side of the cells. In the same experiment, six wells were not pre-exposed to BSO and acivicin, and the uptake of 1 mM [^{3}H]-GSH (0.1 μCi) in PBS^+ for 5, 10 and 20 min, was measured, again via the upper or the lower side of the cells.

The influx of 1 mM [^{3}H]-GSH was also studied in rat and human type II cells in suspension either immediately after their isolation (purity 85%) or after 48 h in culture (purity >95%). In the latter instance the cells were detached from the culture plates with 0.25% trypsin for 5 min.

All these influx studies were not only done at 37°C, but also at 4°C (on ice). To determine the contribution of nonspecific binding of [^{3}H]-GSH to the plastic culture plates or the type II cells, wells with or without type II cells were exposed to [^{3}H]-GSH and immediately washed three times with PBS^+. These values were subtracted from the "uptake" values. After incubation, influx was terminated by washing twice with 200 μl ice-cold PBS^+ buffer, followed by incubation at 4°C with 200 μl of 5% BSA for another 5 min to displace surface-bound radioactivity, which was followed by three more washes at 4°C. This procedure was followed to reproduce as closely as possible the procedures followed by others.[9–14,19] Next, the cells were dissolved in 200 μl NaOH (0.1 M) for at least 3 h; 4.5 ml emulsifier scintillant were added, and radioactivity was measured by liquid scintillation spectrophotometry.

GSH Efflux Studies in Rat and Human Adherent Type II Cells

Glutathione efflux measurements were carried out as described by Sze *et al.*,[20] in cells 48 h after isolation. In these experiments the cells were also pre-exposed to 250 μM acivicin and 1 mM BSO for 30 min, and they were then washed with PBS^+ and incubated in PBS^+ without any addition (control) or with 1 mM DTT, 2 mM GSH, 2 mM GSSG, 2 mM cystine, 2 mM penicillamine or 2 mM N-acetylcysteine for 60 min at 37°C. Then, intracellular GSH levels were measured, as described below, after washing the cells three

times with PBS^+ buffer. Another set of cells was washed five times with pre-warmed PBS^+ buffer, and finally 100 µl PBS^+ was added to the cells. This supernatant was removed after 5, 20, 40 and 60 min for GSH determination. After the extensive washings cell viability was determined by exclusion of trypan blue. The same experiments were performed at 4°C, and efflux was defined as the difference between the efflux at 37°C and 4°C. As the accumulation of GSH in the supernatant was linear, efflux rates were calculated by linear regression.

GSH Determination

The sum of GSH and GSSG was determined using glutathione-reductase according to Anderson,[21] which was adapted as described before.[8] The protein content in each 96-well was measured by the method of Bradford.[22]

Analysis of Data

The experiments were performed on cells obtained from at least three animals and five human lung specimens. Approximately, 10–15 × 10^6 type II cells were obtained per rat, and about 0.5 × 10^6 type II cells/g human lung. All results are expressed as means ± SD. Statistical analysis was performed by Student's paired *t*-test and ANOVA using the SAS/STAT package (6th version). The level of significance was set at $p < 0.05$.

RESULTS

GSH Influx

Exp. (a) In rat type II cells the initial GSH influx rate was 0.70 ± 0.20 nmol/min/mg protein (37°C) and 0.35 ± 0.04 nmol/min/mg protein (4°C, $p < 0.05$) during the first 5 min followed by a minimal increase (Figure 1). In human type II cells, the initial GSH influx rate was 0.36 ± 0.30 nmol/min/mg protein (37°C) and 0.32 ± 0.06 nmol/min/mg protein (4°C) during the first 10 min. Thereafter, no further influx, or even a tendency of the type II cells to lose radioactivity was found.

Exp. (b) Compared to Exp. (a) pre-exposure to cystine led to a GSH uptake which was 1.6, 1.5 and 1.7-fold higher after 10, 15 and 30 min, respectively, in rat type II cells ($p < 0.05$), and 2.2, 2.1, 1.9 and 1.6-fold higher after 5, 10, 15 and 30 min, respectively, in human type II cells ($p < 0.01$). DTT pre-exposure did not affect the results in rat type II cells, but it significantly decreased the influx in human type II cells after 15 min (1.6-fold) and after 30 min (1.4-fold) ($p < 0.05$).

Exp. (c) No uptake was found after subtraction of the influx at 4°C (data not shown).

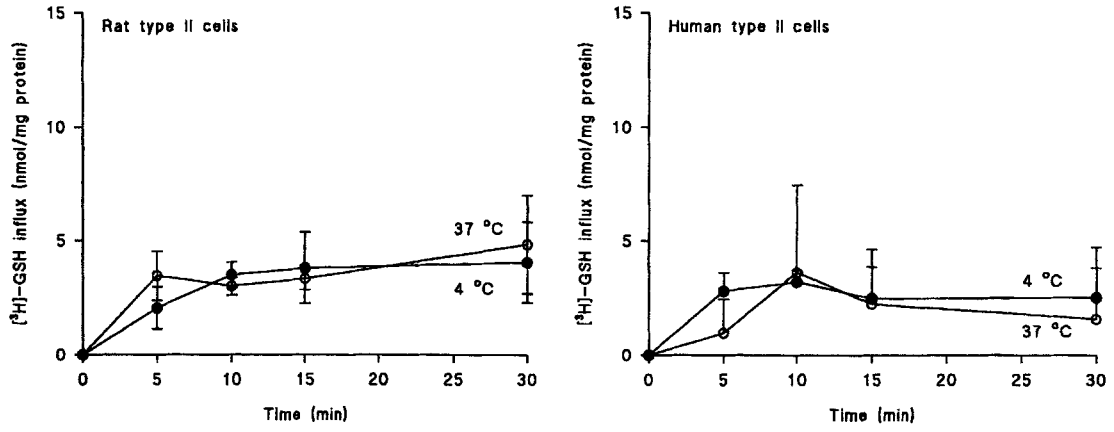

FIGURE 1 Influx of [^{3}H]-GSH in rat and human type II cells. Rat ($n = 3$) and human type II cells ($n = 5$) were pretreated with acivicin (250 µM) and BSO (1 mM) for 30 min and then exposed to 1 mM [^{3}H]-GSH at 4°C or 37°C (means ± SD).

Exp. (d) A linear increase in GSH influx without saturation or difference between the uptake at 4°C or 37°C was found, both for rat and human type II cells. The apparent "knick" in the curve at 20 mM is presumably due to experimental variability, and not significantly different from the uptake at 4°C (Figure 2).

Exp. (e) No net uptake was found in these four test conditions (data not shown).

Exp. (f) Ouabain had no significant effect on the influx of GSH, although there tended to be a decrease at both temperatures (data not shown).

Inserts were also used to investigate the influence of the polarity of the cells. After subtraction of non-specific binding to the membrane and the influx at 4°C, however, no net influx could be detected, neither at the apical side, nor at the basolateral side. In contrast, cells not pre-exposed to BSO and acivicin showed a net GSH uptake which was higher at the basolateral side than at the apical side (Figure 3).

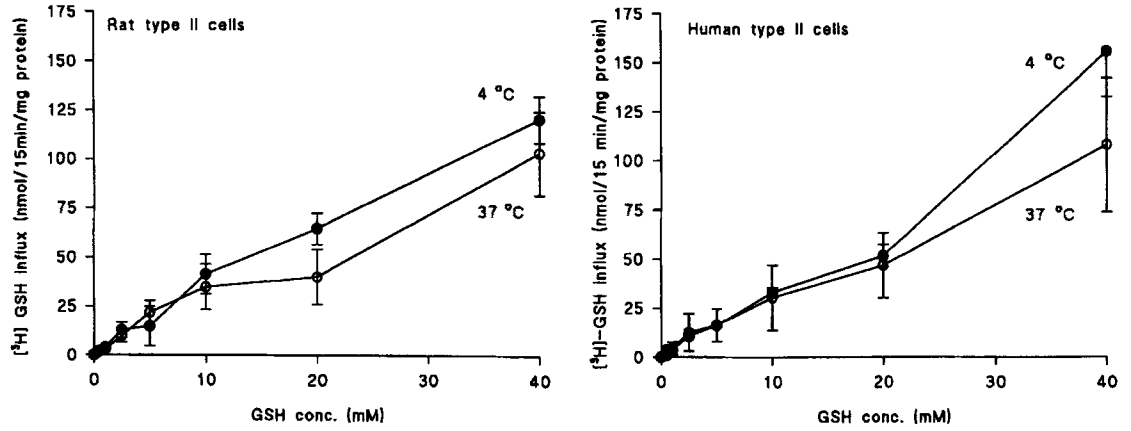

FIGURE 2 Influx of different concentrations [^{3}H]-GSH in rat ($n = 3$) and human type II cells ($n = 5$) pretreated with acivicin (250 μM) and BSO (1 mM) for 15 min at 4°C or 37°C (means ± SD, $n = 3$).

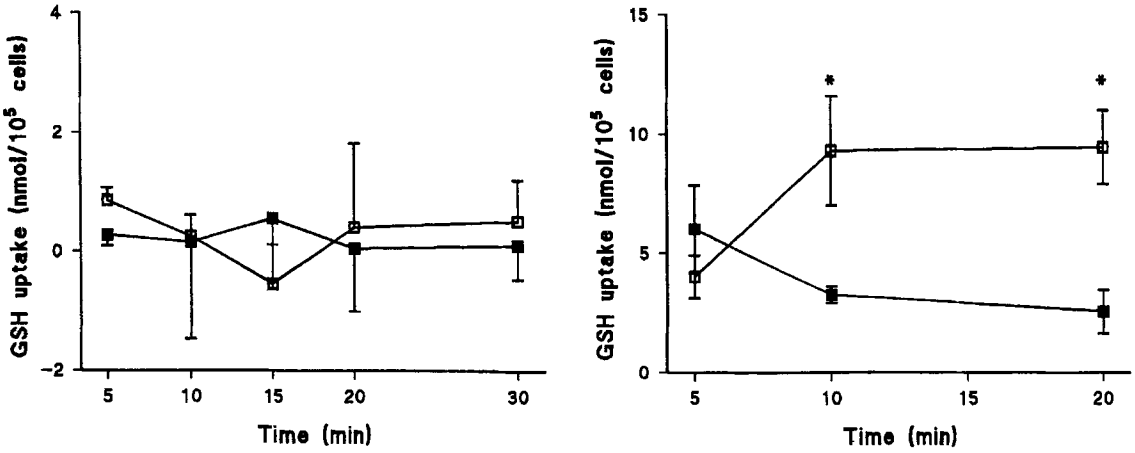

FIGURE 3 Influx of 1 mM [^{3}H]-GSH via the apical side (open squares) or the basolateral side (solid squares) in rat type II cells cultured on inserts and pretreated with acivicin (250 μM) and BSO (1 mM) for 30 min (left side), or without pretreatment with acivicin and BSO (right side) (*$p < 0.05$, paired t-test, means ± SD, $n = 2$).

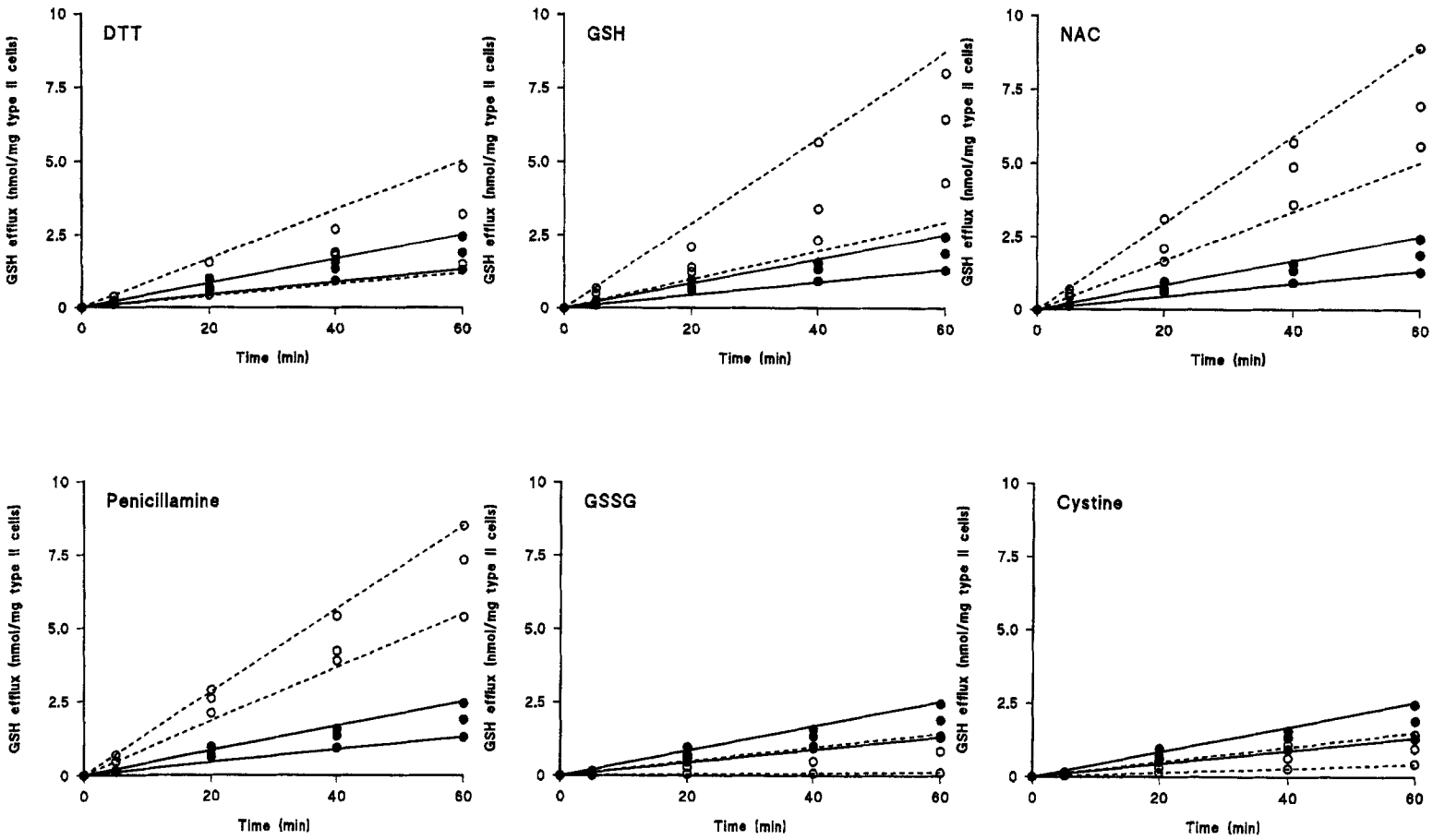

FIGURE 4 Effect of pre-exposure to various reduced and oxidized thiols on GSH efflux from cultured rat type II cells. Solid lines: upper and lower 95% C.I. of the control cells. Dashed lines upper and lower 95% C.I. of the pretreated cells. Efflux rates were calculated by linear regression. Significant differences in the regression coefficients compared to the untreated control cells were found for pre-exposure with glutathione (GSH, $p < 0.05$), N-acetylcysteine (NAC, $p < 0.01$), penicillamine ($p < 0.01$), oxidized glutathione (GSSG, $p < 0.05$) and cystine ($p < 0.05$) ($n = 3$, ANOVA, post-test Dunnett). DTT = dithiothreitol.

The influx of 1 mM [^{3}H]-GSH in freshly isolated rat type II cells in suspension was 2.3 ± 0.3 nmol/mg protein after 15 min at 37°C, and 2.8 ± 0.2 nmol/mg protein at 4°C. For the freshly isolated human type II cell suspension, the influx after 15 min was 1.2 ± 0.3 nmol/mg protein at 37°C and 1.0 ± 0.3 nmol/mg protein at 4°C. There was no uptake either in re-suspended cells having grown on culture plates for 48 h (not shown).

GSH Efflux

The effects of pre-exposure of the cells to reduced thiols (DTT, GSH, NAC, penicillamine) or oxidized thiols (GSSG, cystine) on the efflux of GSH are summarized in Figure 4 for the rat type II cells, and in Figure 5 for the human type II cells. Cell viability remained >95% despite the exposure to the various agents and extensive washings. Exposure to the reduced or oxidized thiol compounds slightly increased intracellular GSH levels, but these increases did not reach the level of significance. All reduced thiol compounds used led to an increased efflux (GSH accumulation in the supernatant), which was linear as a function of time at 37°C, but not at 4°C. In contrast, the oxidized thiols decreased efflux (except GSSG in human type II cells).

DISCUSSION

In both rat and human type II cells no arguments were found for an active uptake of [^{3}H]-GSH, mainly because the uptake was temperature-independent and because it appeared to be non-saturable up to 40 mM. When early kinetics were done, no uptake was found either. The polarity of the cells did not appear to influence the results. Pre-exposure of the type II cells to cystine increased GSH influx in rat and human type II cells, while DTT tended to decrease the influx in rats, and significantly decreased it in human type II cells. GSH efflux was favoured by pre-incubation with reduced thiol compounds (DTT, GSH, NAC, penicillamine). In contrast, pre-incubation with oxidized thiols (GSSG, cystine) tended to decrease GSH efflux.

Interpretation

Since 1986, several authors, mostly from the same research group, have proposed the existence of an uptake of intact GSH by rat and rabbit type II pneumocytes, kidney cells and small intestine.[9–14] They studied the uptake of one GSH concentration as a function of time, but the kinetics of this uptake have not been investigated, except in retinal epithelial cells[19] and in Hep G2 cells.[20] The reported uptake rates of GSH were rather variable, even when the same cell types were compared: e.g. for rat type II cells Bai *et al.*[9] found an uptake of 75 nmol/10^6 cells/10 min, while Hagen *et al.*[12] found no more than 5 and 8 nmol/10^6 cells/15 and 30 min, respectively, when the type II cells were incubated with 1 mM GSH. In two studies a (sub)-physiological concentration of 0.02 mM was used – normal GSH levels in the epithelial lining fluid are 0.4 mM and in these studies the differences in measured uptake are even more striking: Brown *et al.*[10] found an uptake of 20 nmol/10^6 cells/5 min in the rabbit, while Jenkinson *et al.*[14] found only 4.8 pmol/10^6 cells/5 min. No uptake of intact GSH was found in liver cells, fibroblasts and (pulmonary) endothelial cells.[22]

In the present study, however, no evidence for an active energy-dependent uptake mechanism (Figures 1 and 2) was found. One might object that the uptake mechanism could have been destroyed by the trypsin used for the isolation procedure, but this would be less applicable for healthy-looking cells in monolayers 48 h after their isolation, and for resuspended cells detached after 48 h in culture with a very weak trypsin solution for only 2 min. It is also possible that type II cells only take up intact GSH via the basolateral membrane, as has been demonstrated for kidney cells and hepatocytes,[5,11] because

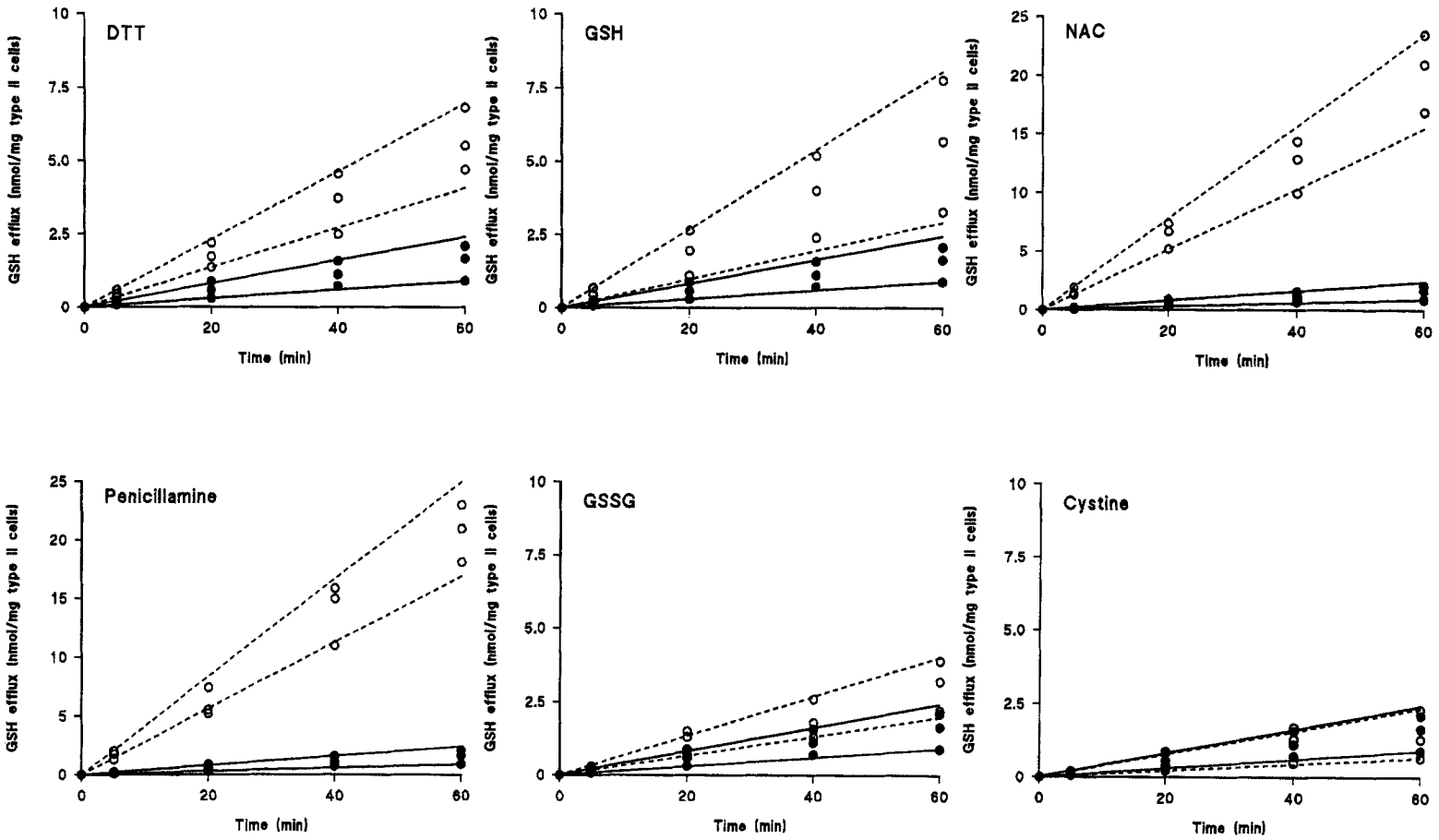

FIGURE 5 GSH efflux for human type II cells (for symbols see Figure 4). Significant differences in the regression coefficients compared to the untreated control cells were found for pre-exposure with dithiothreitol (DTT, $p < 0.01$), glutathione (GSH, $p < 0.01$), N-acetylcysteine (NAC, $p < 0.01$), penicillamine ($p < 0.01$), and cystine ($p < 0.05$) (ANOVA, post-test Dunnett). GSSG = oxidized glutathione.

pulmonary vascular endothelial cells contain active mechanisms to selectively transport certain macromolecules, including possibly GSH, by transcytosis via the interstitium into the alveolar epithelium.[24] This question was addressed by studying cells grown on inserts allowing contact with medium from the basolateral side. However, these experiments again failed to demonstrate active uptake of GSH via the basolateral membrane in the presence of BSO and acivicin (Figure 3). This was not due to any inadequacy of our experimental system, because in the absence of BSO and acivicin significant GSH was apparently "taken up" via the basolateral membrane (Figure 3, right panel), in accordance with the results reported for kidney cells and hepatocytes due to breakdown by γ-GT and intracellular resynthesis of GSH.[5,11] The only explanation we have for the discrepancy between the results of our study and those of other investigators, is that most of them did not study passive accumulation (e.g. uptake at 4°C) or subtract non-specific binding of the labelled GSH to the cell membrane or the plastic of the wells, thus leading to an artifactual uptake. We studied the influx of GSH in type II cells in suspension according to the same experimental protocol as did others,[10–12] including pre-oxygenation and a Krebs buffer, but no GSH uptake was found. Assuming that the intracellular water space of 10^6 type II cells is about 0.33 µl,[25] the basal intracellular GSH concentration will be about 1 mM, but lower than 1 mM after pre-exposure to BSO plus acivicin. This might allow extracellular GSH levels higher than at least 1 mM to increase intracellular GSH concentrations by simple equilibrium, but it might also explain why neither Jenkinson *et al.*,[14] nor we, could demonstrate significant influx at physiological or sub-physiological extracellular GSH concentrations.

There is increasing evidence that GSH is exported from the cell and that this pathway is dependent on the redox status of the extracellular environment.[5–7] Deneke and coworkers[23] found that incubation of lung type II cells with RPMI medium without cystine resulted in a dramatic and rapid decline in intracellular GSH levels. They suggested that exogenous cystine, by causing a more oxidized environment, might block transport of GSH out of the cell. In the present study we demonstrated that pre-exposure of rat type II cells to the oxidized thiol compounds, cystine and GSSG, significantly diminished GSH efflux (2.3- and 2.9-fold, respectively), but in human type II cells this decrease (1.3-fold) was only significant after exposure to cystine (we have no explanation for the discrepant effect regarding GSSG in the human cells). The effect of pre-exposure to reduced thiol compounds on GSH efflux was very pronounced with up to a 3-fold increase in rats and a 10-fold increase in GSH efflux in human type II cells (Figures 4 and 5). We also found that the transporter is not completely symmetrical: after pre-exposure to DTT a lower uptake of [^{3}H]-GSH was found, while pre-exposure to cystine significantly increased the uptake. Efflux of GSH was thus stimulated and influx inhibited by pre-exposure of the cells to reduced thiols, while disulphides inhibited the efflux favouring inward transport. Very recently, the GSH transporter has probably been identified as the cystic fibrosis transmembrane conductance regulator (CFTR) transporter protein.[26] The results of that study suggest that CFTR, in addition to its role as a Cl^- channel, may function as a permeation pathway by which GSH and GSSG are released into the epithelial lining fluid. The CFTR transporter protein appeared to function under physiological conditions mainly as an effluxer for GSH and GSSG, which is consistent with the results in the present study. As CFTR mRNA has been demonstrated in type II pneumocytes, the previously unknown GSH transporter has probably been identified,[27] but further studies in this direction are certainly warranted.

Painstaking efforts were made to reproduce findings by others that intact GSH can be accumulated by an active process in type II pneumocytes. We believe that all possible experimental

factors were investigated, and conclude that there is no uptake of intact GSH by primary type II cells in culture, neither from rat, nor from human. What we did find was that efflux and influx of GSH are influenced by the extracellular redox state.

CONCLUSION

Our data indicate that in human and rat type II pneumocytes a GSH-carrier exists which operates as an effluxer. At GSH concentrations in the physiological range no uptake is seen but some uptake can be observed at GSH concentrations above normal physiological levels. The uptake appeared to be energy-independent and non-saturable. Efflux of GSH was stimulated and influx inhibited by pre-exposure of the cells to reduced thiols, while disulphides inhibited the efflux favouring inward uptake. GSH uptake in type II cells thus may depend on concentration gradients and driving forces, such as the redox state of the extracellular fluid, and share some of the characteristics of the sinusoidal GSH transporter in the liver.

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