

REVIEW

Cellular glutathione turnover *in vitro*, with emphasis on type II pneumocytes

R.J. van Klaveren, M. Demedts, B. Nemery

Cellular glutathione turnover in vitro, with emphasis on type II pneumocytes. R.J. van Klaveren, M. Demedts, B. Nemery. ©ERS Journals Ltd 1997.

ABSTRACT: The most important extracellular antioxidant in the lung is glutathione (GSH). The epithelial lining fluid of normal lungs contains very high concentrations of this tripeptide, about 100 times higher than that found in the extracellular fluid of many other tissues. How these high extracellular GSH levels are established and the mechanisms for increases (*e.g.* smokers) or decreases (*e.g.* lung fibrosis) are still unknown, but more insight into the regulation of GSH turnover in type II pneumocytes has recently become available.

The purpose of this review is to give an overview of the literature concerning cellular GSH turnover for different cell types *in vitro*, with an emphasis on alveolar type II epithelial cells. The main messages of this review are that: 1) GSH is, in fact, an important vehicle for stabilizing, detoxifying and transferring cysteine; 2) cysteine is the rate-limiting substrate for GSH synthesis, especially under conditions of oxidative stress; 3) various transport systems exist for the uptake of the constituents of GSH, of which γ -glutamyltransferase appears to be important; 4) intracellular GSH levels of the type II cells are governed by different factors, including, probably, the extracellular redox state; and 5) a more reduced extracellular redox state appears to favour GSH efflux, whilst an oxidized state leads to retention of GSH inside the cell.

These concepts should lead to reconsideration of some of the conventional approaches to increasing intracellular glutathione levels.

Eur Respir J 1997; 10: 1392–1400.

It is evident that the lung is a primary target organ for oxidant injury because of its large surface and its exposure to oxygen and many other inhaled toxic oxidants, such as ozone and cigarette smoke, as well as to endogenously released oxidants during pulmonary inflammation. It is, therefore, easy to understand that the lung needs a strong extracellular antioxidant defence system to prevent cellular attacks by reactive oxygen species, and damage to the alveolar epithelial cells. The most important extracellular antioxidant in the lung is glutathione (GSH) [1]. Although the extracellular concentration of this peptide is usually low, the alveolar epithelial surface of the lung is different in this regard: the epithelial lining fluid (ELF) of normal lungs contains a very high concentration (about 400 μ M) of GSH, *i.e.* 100 times higher than that found in the extracellular fluid of many other tissues [1, 2]. Moreover, the extracellular fluid in the alveolus of the lung also contains antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione reductase and glutathione peroxidase, thus confirming the existence of a very strong antioxidant defence screen [3].

How these high extracellular GSH levels are established is still unknown, but simple diffusion from blood plasma may be excluded, because blood plasma GSH levels are much lower (0.5–5 μ M). Proposed mechanisms are increased release from intracellular stores because of increased membrane permeability, cell lysis, or

active transport [2]. It has been observed that the GSH levels of ELF change in certain conditions. Exposure to hyperoxia for several days, for example, increases ELF GSH levels [2], and tobacco smokers also show an increase in ELF GSH, with concentrations up to almost twice the normal level [1]. On the other hand, ELF GSH concentrations have been shown to decrease in diseases such as interstitial pulmonary fibrosis (in which ELF GSH is fourfold lower than in healthy subjects) [4], cystic fibrosis [5], symptom-free human immunodeficiency virus (HIV)-positive individuals [6], and in adult respiratory distress syndrome (ARDS) [7, 8]. Although it appears that certain disease states are correlated with changes in GSH in the ELF, the underlying mechanisms for these changes are still far from being understood, and it is unknown whether they are the cause or the consequence of the disease.

Recently, many studies have been performed on the epithelial type II pneumocytes, to elucidate the potential role of these cells in the regulation of cellular GSH turnover in the lung. Although these cells make up only 5% of the surface area of the lung, and represent only 10–15% of the total lung cell population [9], they represent an important cell population in the lung, because they are metabolically very active. The most important functions of these cells are the synthesis and secretion of pulmonary surfactant, and the ability to function as

Laboratory of Pneumology, Unit of Lung Toxicology, K.U. Leuven, Leuven, Belgium.

Correspondence: B. Nemery
Pneumology K.U. Leuven
Herestraat 49
B-3000 Leuven
Belgium

Keywords: Cysteine
cystine
 γ -glutamyltransferase
glutathione
transport systems
type II pneumocytes

Received: August 29 1996
Accepted after revision January 22 1997

Supported in part by the Belgian NFWO, the Commission of the European Communities (Biomed programme) and the "Dr P. Tuybens Leerstoel in Toxicologie".

stem cells to renew the epithelium in the event of type I cell injury [10]. Although the type I pneumocytes make up 90–95% of the alveolar epithelial surface of the lung, they account for only 4–5% of the total lung cell population [9]. Their main function is to line the alveolar surface and to provide a barrier for gas diffusion. They are much more difficult to study and their metabolic functions, especially with respect to GSH metabolism, are much less prominent than those of type II cells, or are probably even absent. For example, γ -glutamyltransferase (γ -GT), an enzyme that plays an important role in the breakdown of extracellular GSH, is only present in very low concentrations in type I cells [11]. Because of their large surface and low metabolic activity, it is generally assumed that type I pneumocytes are more susceptible to injury and oxidative stress than type II pneumocytes.

Several excellent reviews are available on the mechanism and pathophysiological consequences of oxidant exposure [12, 13], the role of GSH as one of the most important endogenous antioxidants [14–17], and the potential therapeutic use of sulphhydryl (SH) compounds (N-acetylcysteine) in protecting the lung against oxidative damage [3]. The purpose of this review is to provide an overview of the literature concerning cellular GSH turn-over for different cell types *in vitro*, an issue which has not received much attention so far. In particular, we will discuss the different mechanisms involved in GSH metabolism in type II cells and the role of the extracellular redox state in antioxidant defence, and we will search for similarities and differences with other cell types, in order to highlight gaps in our knowledge, which might be of value for further research. Furthermore, we will reassess, with respect to our present insights in cellular GSH turnover, existing strategies for augmenting intracellular GSH levels.

Glutathione in perspective

The tripeptide GSH (γ -glutamyl-cysteinyl-glycine) (fig. 1) was isolated from yeasts by Rey-Pailhade in 1888, but it really found a place in biochemistry through a report of Hopkins in 1921. The correct structure was established by synthesis in 1935 [18]. GSH is widely-distributed in all living organisms and is found predominantly inside cells, where it reaches relatively high concentrations (0.1–10 mM) [14]. In contrast, plasma concentrations of GSH are usually in the low micromolar range, but they may vary widely. GSH accounts for 90% of the intracellular nonprotein thiols, and is, therefore, the most important intracellular reducing agent [14]. The liver is the main source of GSH, and virtually all GSH newly synthesized in the liver is exported. Most of the circulating GSH originates from the liver, and it has been estimated that approximately 45 mmol (or 14 g) GSH is released into the circulation in humans during 24 h [19]. The lungs, like the liver and the kidney, also show a high rate of GSH turnover, and might even be a greater importer of GSH than the kidney. Plasma half-life of GSH is less than 2 min, and cellular half-life has been determined to be about 3–4 h [20]. Many biological functions have been ascribed to GSH, including: 1) maintenance of SH groups in a reduced state in proteins and other molecules; 2) destruction of hydrogen peroxide, other peroxides and free radicals; 3) catalysis of disulphide

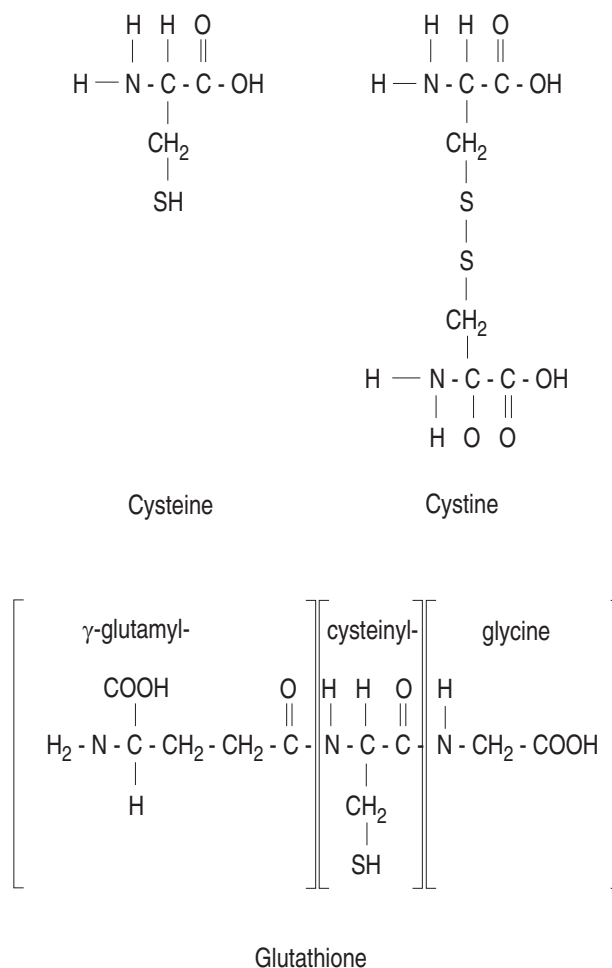


Fig. 1. – Structure of cysteine, cystine and glutathione.

exchange reactions; 4) acting as a co-enzyme for certain enzymes (*e.g.* glyoxalase); 5) detoxification of foreign compounds by conjugation with GSH; and 6) translocation of amino acids across cell membranes [21]. Although this list of proposed functions is far from complete, it may be stated, in general, that GSH plays a key role in cell defence, and serves as a reservoir for cysteine.

There are now sufficient data to strongly implicate free radical injury in the genesis and maintenance of several lung disorders in humans, and to state that restoration of intracellular GSH is correlated with increased antioxidant defence [11–16]. In the light of our aim to augment antioxidant capacity of (lung) cells, in order to increase their resistance against various kinds of oxidative stress, most investigators have focused on and searched for methods to increase intracellular GSH levels, mostly by administration of reduced thiol compounds. Relatively little attention has been paid to the role of the redox state of the extracellular environment on intracellular GSH levels. Yet, the extracellular redox state has an important regulatory role with respect to the import and export of GSH, as will become clear from this review. Since there are considerable differences between the various cell types regarding the uptake of intact GSH and GSH precursor molecules, our treatment strategy to augment antioxidant defence should probably be differentiated and adapted to the target organ or cells that we intend to protect. Cellular GSH turnover

is rather complex, and our knowledge of it is far from complete, especially with regard to the type II pneumocytes. Nevertheless, certain conclusions obtained from the study of other cell types might also apply for type II cells.

Intracellular GSH levels: a balance between influx, synthesis, efflux and consumption

When studying GSH turnover in different kinds of cells, it is important to remember that GSH is in fact "a vehicle for stabilizing, detoxifying and transferring cysteine" [13]. Thus, the study of GSH turnover also implies a study of cysteine/cystine transport, because most of the intracellular GSH is the result of the transfer of its constituents (cysteine, glutamine and glycine) into the cell. The level of intracellular GSH is, in fact, the final outcome of a complex process, which can, for practical reasons, be divided in: 1) uptake of constituents of the GSH molecule *via* amino acid uptake pathways or *via* the γ -GT pathway; 2) uptake of intact GSH; 3) intracellular GSH synthesis and degradation; and 4) the efflux of intact GSH and oxidized GSH (GSSG) [13]. For type II pneumocytes, the different pathways are illustrated in figure 2. These different routes will be discussed consecutively.

Amino acid transport systems and GSH synthesis (fig. 3)

Cysteine and cystine are nonessential amino acids, synthesized primarily in the liver from methionine *via* transsulphuration pathways [22]. The activity of transsulphuration differs in isolated liver cells from the *in vivo* situation, in that isolated liver cells (like other cell types *in vitro*) usually lack the transsulphuration pathway, and thus require exogenous cysteine or cystine [22]. Cysteine is one of the three components of GSH, and the rate-limiting substrate for GSH synthesis in various cell types in culture, especially under conditions of oxidative stress [22–24]. Glutamate or glycine are rarely rate-limiting [22, 25]. The availability of cysteine or cystine has been reported to be strongly correlated with intracellular GSH levels [24–30]. However, it is not only the presence of extracellular cysteine or cystine that is important in the rate of intracellular GSH synthesis, but also the extracellular redox state (which is in part determined by extracellular GSH levels), which influences the uptake of

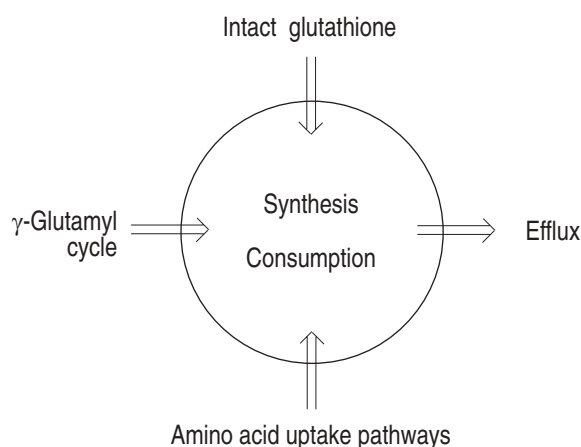


Fig. 2. – Schematic representation of glutathione (GSH) metabolism in type II pneumocytes.

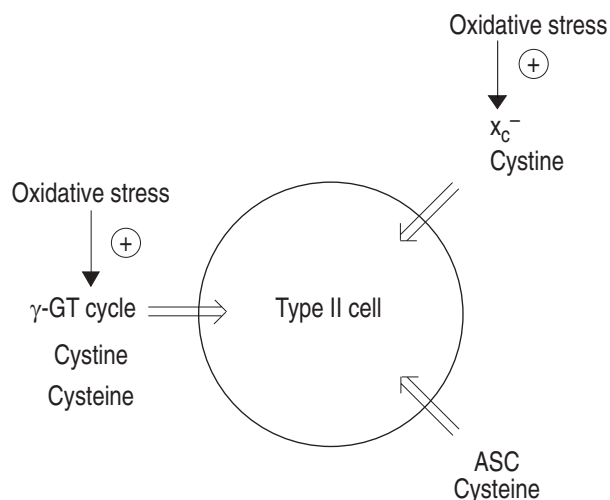


Fig. 3. – Schematic representation of the uptake pathways for cysteine and cystine in type II pneumocytes. The ASC system is non-inducible; γ -glutamyltransferase (γ -GT) and the x_c^- pathway might be induced by oxidative stress.

cysteine/cystine, used for intracellular GSH synthesis [25, 31]. The different amino acid transport systems have been labelled A, ASC and L by CHRISTENSEN [32]. The A and ASC systems are sodium-dependent and are used for the uptake of neutral amino acids with small side chains, such as cysteine, whilst the L system is sodium-independent and specific for neutral amino acids with large branched and apolar side chains, such as methionine [22]. In most cells, the ASC system is the major constitutive transport system, *i.e.* not inducible by amino acid starvation or oxidant stress. As long as cysteine is present extracellularly, its intracellular level may be maintained by the ASC transport system. The characteristics of cysteine transport are not significantly different in type II cells from those reported in other cell types [33], but it is important to realise that in endothelial cells, fibroblasts, and type II pneumocytes, cysteine transport is more efficient than cystine transport, which cannot be taken up *via* the ASC system because it is an anionic amino acid [25, 31]. Intracellular cysteine levels could, thus, be increased either by reduction of extracellular cystine to cysteine, *e.g.* by addition of extracellular GSH, N-acetylcysteine, or cysteamine, or by increasing the transport of cystine *via* the x_c^- system (see below). The latter is feasible because cystine transport is inducible, in contrast to the ASC system [34].

Cystine is taken up into the cell *via* the x_c^- system, as described by BANNAI and co-workers [35, 36] for human fibroblasts, neuronal cell cultures [22], hepatocytes [22, 37], human umbilical vein endothelial cells (HUVEC) [22], mouse peritoneal macrophages [38], and, recently, also for human pulmonary endothelial cells [39]. This system is specific for anionic amino acids, and is strongly inhibited by glutamate [35]. Two kinds of x_c^- uptake systems have been described: a sodium-dependent and a sodium-independent one [37]. In the liver, both transport systems have been found, but only the sodium-independent system was inducible [37]. In bovine pulmonary artery endothelial cells (BPAEC), the x_c^- system was found to be sodium-independent and inducible by diethyl maleate (DEM), arsenite and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [23, 24, 39, 40]. In human fibroblasts

and HUVEC, these agents have been shown to induce cystine uptake twofold in the first 30 min, after 60 min preincubation with 25 μ M DEM [26].

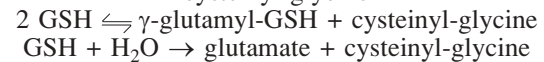
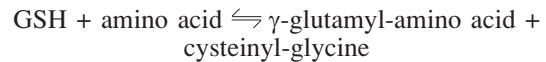
The x_c^- system in type II cells might be different from the system described in endothelial cells, hepatocytes and fibroblasts, in that no inducible cystine transport system could be demonstrated, but only the sodium-dependent, noninducible system [33]. However, despite the absence of an inducible cystine transport system, treatment with DEM did increase intracellular GSH levels in type II cells, but the mechanism for this increase remains to be determined. The x_c^- system in type II cells also differed from the x_c^- system described previously in BPAEC or human fibroblasts, in that the pattern of inhibition by anionic and neutral amino acids in type II cells was different [33].

The mechanism for the induction of the cystine transport system is not completely understood [40, 41]. It has been shown that overnight incubation of HUVEC cells in a medium lacking sulphur (M199-medium) depleted GSH by more than 70% and increased cystine uptake over the first 60 min by up to 300% [42]. This effect disappeared when the cells were co-incubated with cycloheximide, and the induction of the cystine transport *via* the x_c^- system was not seen when the cells were treated with L-buthionine-[S,R]-sulphoximide (BSO) [40], which suggests that GSH depletion *per se* is not critical for the induction of this transport system in "sulphur-starved" cells. However, an increase in uptake of cystine *via* the γ -GT pathway was demonstrated in response to pretreatment with the M199-medium [40]. These findings might fit with our own findings of an increase in γ -GT activity due to GSH depletion in type II cells [43]. The level of glutathione transferase in cells is associated with the susceptibility of the cell or organ to depletion of GSH by exogenous electrophilic agents. In the liver, where glutathione transferase levels are exceptionally high, administration of agents, such as bromobenzene, phorone or DEM, results in rapid depletion of GSH [14].

γ -glutamyltransferase and intra- and extracellular GSH levels

The enzyme γ -GT is an apical membrane protein present in many epithelial cells [44]. Although synthesized as a single polypeptide, γ -GT is cleaved to form a large 55 kDa membrane

anchor subunit and a smaller catalytic subunit [44]. γ -GT plays a key role in the γ -glutamyl cycle, a pathway for the synthesis and degradation of GSH. γ -GT is a plasma membrane enzyme, with its active site directed toward the outside of the cell [45]. This enzyme is the only enzyme that can break the γ -glutamyl bond of GSH, thus releasing cysteinyl-glycine [44–46]. The glutamyl moiety is then transferred to an amino acid, a dipeptide or GSH itself, producing the respective γ -glutamyl derivatives (fig. 4):



The γ -glutamyl-amino acid is taken up into the cell, where it is cleaved to free amino acids and glutamate [46]. The extracellular cysteinyl-glycine part of the GSH molecule is either hydrolysed by a dipeptidase (cysteine and glycine are then taken up separately into the cell), or transported as cysteinyl-glycine into the cell [47].

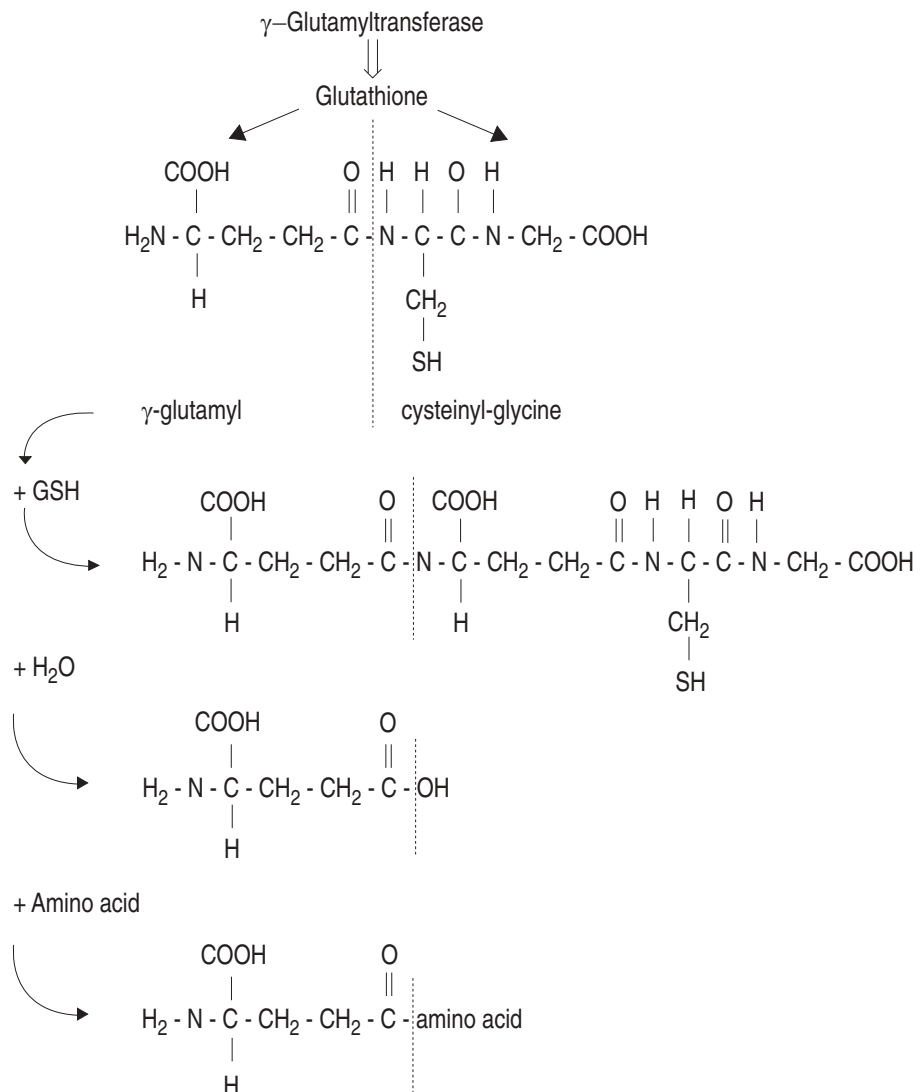


Fig. 4. — Action of γ -glutamyltransferase (γ -GT) and the respective γ -glutamyl derivatives, γ -glutamyl-GSH, glutamate and γ -glutamyl-amino acid. GSH: glutathione.

This series of enzyme-catalysed reactions, along with the two steps for *de novo* GSH synthesis, has been called the γ -glutamyl cycle (fig. 5).

γ -GT has been localized on the luminal surface of rat Clara cells and type II cells [11, 48]. Although even higher immunoreactivity was associated with type I pneumocytes by indirect immunofluorescence and Western blot analysis, it is not certain that this activity originates from the type I cells, because type II cells lose their γ -GT when they progress towards type I cells in culture, suggesting that much of the immunoreactive γ -GT of the type I cell is probably nonfunctional [48, 49]. One explanation is that the type II cells, not only synthesize a γ -GT protein, but also release it, probably together with phosphatidylcholine, which associates with surfactant [50]. It is likely to be this association of γ -GT with surfactant that accounts for the apparent immunocytochemical localization on type I cells and macrophages [49, 51]. Another possible explanation is that type I cells express only the larger uncleaved form of γ -GT, explaining the high level of immunoreactivity, but also the low specific activity [48]. The functionality of this type I cell associated γ -GT is, however, incompletely defined. The surfactant associated γ -GT could function to regulate the size of the intra-alveolar GSH pool. Hence, it could influence the ability of GSH to function as an extracellular reducing agent within the alveolus, and, therefore, also influence the export of GSH out of the cell (see below), or it could provide a means for the production of γ -glutamyl-amino acids within the alveolus. The acidic pH of the alveolar lining fluid would favour the γ -GT catalysed hydrolysis reaction over the transpeptidation reaction, and the split products could serve as substrates for GSH synthesis by the cells in the alveolar space [52]. Dipeptidases, which complete the hydrolysis of cysteinyl-glycine have also been demonstrated in the alveolus [53].

Besides the breakdown of extracellular GSH and, less efficiently, of GSSG, γ -GT has a role in several physiological and toxicological processes. It removes, for example, the glutamate moiety from glutathione conjugates, catalyses the conversion of leukotriene C₄ (LTC₄) to leukotriene D₄ (LTD₄) and the conversion of glutathione conjugates to cysteine-S conjugates [54, 55]. Recent studies with endothelial cells, alveolar macrophages and fibroblasts have shown that γ -GT might play a role in the

protection against oxidative stress, since specific γ -GT inhibitors (serine-borate complex and acivicin) can prevent the protective effect afforded by extracellular glutathione against added oxidants [43, 56–58]. On the other hand, after murine fibroblasts had been depleted from intracellular GSH with DEM, the recovery of intracellular GSH was increased, compared to control cells, if γ -GT activity had been increased by transfection with recombinant γ -GT [57]. As far as we know, there have only been two reports on the induction of γ -GT activity due to oxidative stress in type II cells [43, 58]. In the first study [58], exposure of a lung L2 epithelial tumour cell line to the oxidant menadione gave a dose-dependent induction of γ -GT activity, amounting to 2–8 times the control values. In our own study [43], we demonstrated that, in freshly isolated rat type II cells, increases in γ -GT activity were governed more by GSH depletion than by oxidative stress. Phenobarbital, steroids and xenobiotics are inducers of γ -GT expression in hepatocytes [59], but there is no evidence for induction by these compounds in the lung or in isolated type II cells (own unpublished data).

The γ -glutamyl cycle is also an important pathway for the uptake of cystine. This evidence is mainly circumstantial, but includes the fact that γ -GT possesses a low K_m for cystine, and prefers cystine as an acceptor for transpeptidation of GSH [60]. Cystine is also a potent inhibitor of γ -GT-dependent hydrolysis of GSH. Specific and strong inhibition of γ -GT by acivicin or anthglutin for 6 h in HUVEC resulted in a total depletion of GSH in these cells [40]. This means that intracellular cysteine concentrations were too low, and that the uptake of cystine *via* the x_c⁻ system did not suffice to maintain GSH biosynthesis, thus facilitating the depletion due to continual breakdown and export of GSH [42].

Kidney cells, intestinal epithelial cells and pulmonary type II epithelial cells, in particular, have high levels of γ -GT activity, and are equipped to salvage amino acids from the extracellular GSH pool in their milieu [55]. Endothelial cells, hepatocytes and fibroblasts have lower γ -GT levels, and can, thus, less easily use extracellular GSH for intracellular GSH synthesis [60]. Moreover, in these cells, no transport systems for the cellular uptake of intact GSH have been demonstrated. These differences are very important when comparisons are made between the different cell types with respect to their response to oxidative stress, but also with respect to enhancement of antioxidant defence.

Intracellular GSH synthesis

The rate-limiting step in the synthesis of GSH is γ -glutamyl-cysteine synthetase [21, 45]. The enzyme catalyses the bond between glutamate and cysteine, and is inhibited by BSO [61]. Enzyme activity is regulated by feedback inhibition of GSH, so that GSH, but not GSSG, regulates its own synthesis. In normal steady-state conditions, the enzyme does not function at maximal activity, because patients with 5-oxoprolinuria, who have a GSH deficiency due to a defect in the enzyme glutathione synthetase, overproduce γ -glutamyl-cysteine [45]. The negative feedback of GSH on γ -glutamyl-cysteine synthetase can be circumvented by supplying the cell with high intracellular cystine concentrations (fig. 6) [62]. Cystine

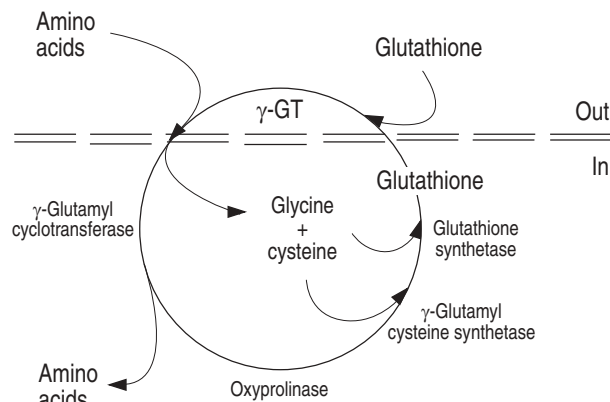


Fig. 5. — The γ -glutamyl cycle: an essential pathway for the cell to maintain adequate intracellular glutathione (GSH) levels. γ -GT: γ -glutamyltransferase.

is directly, or less efficiently after reduction of cystine to cysteine, bound to the γ -glutamyl moiety of GSH, and is taken up into the cell by the action of γ -glutamyl-transferase. The γ -glutamyl-cystine/cysteine formed, thus, bypasses γ -glutamyl-cysteine synthetase, and by the action of GSH-synthetase, higher GSH levels can be achieved.

Glutathione efflux

There is increasing evidence that GSH is exported out of the cell, and that this pathway is an important determinant of the final intracellular GSH level [63–65]. The GSH transporter does not appear to be facilitative and bidirectional, but transport seems to be not perfectly symmetrical, in that the kinetics of uptake differ from the kinetics of efflux [65]. The function of the GSH transporter is influenced by the thiol status of the extracellular environment: in rat hepatocytes it was shown, perhaps contrary to expectation, that high extracellular SH levels (GSH, dithiothreitol and other thiols) promote the efflux of intracellular GSH [63]. In contrast, cystine (and other disulphides, such as GSSG and cystamine), inhibits efflux at physiological concentrations, while promoting the uptake of GSH. Thus, a more oxidized extracellular environment stimulates the cell to retain GSH, while a more reduced extracellular state facilitates GSH efflux [63–65]. The increased release of intracellular GSH in the presence of high extracellular GSH is not simply the result of higher intracellular GSH levels, originating from the breakdown and subsequent uptake of extracellular GSH components by the action of γ -glutamyl-transferase. Indeed, when these cells were incubated with acivicin (to block the degradation of extracellular GSH) and with BSO (to prevent GSH synthesis), they still showed efflux of GSH in the presence of high extracellular GSH levels [63]. The efflux from the liver is also under hormonal control, since vasopressin, phenylephrine and adrenaline stimulate the efflux [66]. These studies were performed on isolated and cultured liver cells only, and so far no studies on the export of GSH have been performed on lung type II cells. DENEKE *et al.* [41] 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 this phenomenon might be explained by the above-

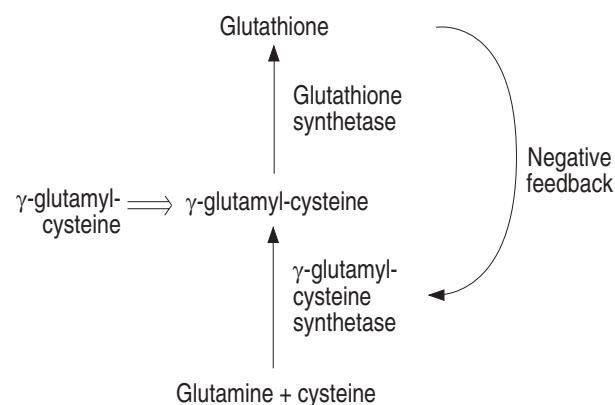


Fig. 6. — Intracellular glutathione (GSH) synthesis, and the possibility to bypass the negative feedback of GSH on the enzyme γ -glutamyl-synthetase by γ -glutamyl-cysteine.

described mechanism, and that exogenous cystine, by causing a more oxidized environment, might block transport of GSH out of the cell. So far, this hypothesis has not been proved.

The ELF in healthy volunteers shows GSH concentrations greater than 400 μ M, which is among the highest reported for any extracellular fluid [1, 2]. Other studies have shown that GSH is present in cerebrospinal fluid, saliva, sweat, seminal fluid and milk. Thus, release of GSH appears to be a general property of cells, which suggests that extracellular GSH has specific biological functions. One of these functions is control of the extracellular redox state, but other functions of extracellular GSH include: acting as a source of cysteine; acting as a substrate for extracellular GSH-dependent enzymes; direct protection of critical thiol groups of transporters and receptors present on the extracellular surface of cells; the reduction of disulphides to control the fluidity of mucus; and the maintenance of other antioxidant systems (*e.g.* ascorbate) [67].

The origin of GSH in the ELF has not been determined, but it is not due to simple diffusion from the plasma, because plasma levels are much lower. Smokers showed an 80% higher GSH concentration in ELF, whereas, in patients with pulmonary fibrosis, GSH concentrations were fourfold lower than in healthy subjects [1, 2]. An explanation for these phenomena is not yet available. Even if a bidirectional GSH transporter existed in the lung, as in the liver, these findings cannot be explained by this mechanism alone: exposure of cells to oxidative stress implicates a more oxidized environment, which should, according to the function of the bidirectional GSH transporter, stimulate the cell to retain GSH inside the cell instead of releasing or exporting GSH into the ELF.

One might speculate that γ -GT, which is located on the extracellular site of the cell membrane, is involved. From a theoretical point of view, surfactant associated γ -GT could function to regulate the size of the intralveolar GSH pool, because γ -GT is the only enzyme that can break down intact GSH. Low γ -GT activity would mean less degradation of the GSH molecule and, thus, higher GSH levels, but in our own study, with rats exposed to hyperoxia, there was no evidence for this: low γ -GT activity in the ELF was strongly associated with low ELF GSH levels [68]. High ELF γ -GT levels in the presence of low ELF GSH levels, and vice versa, might be disastrous because it could lead to either complete GSH depletion or a GSH excess in the ELF.

It seems that ELF γ -GT is not the main determinant in establishing ELF GSH levels, but that a certain ELF GSH level will be the result of a complex interaction between the extracellular redox state, intracellular GSH synthesis (and GSH levels), ELF γ -GT activity, and efflux of GSH from the type II cells into the ELF [68]. Furthermore, the export of GSH from the ELF can be accomplished by a variety of cells, including lymphocytes, macrophages, fibroblasts and endothelial cells, found in large numbers in the lower respiratory tract. It is probable that the consumption of GSH by these cells is different in the aforementioned disease states, but the permeability of the interstitium might also differ and lead to increased or decreased leakage of GSH into the pulmonary circulation [1].

Export of GSSG [69]

In mammalian cells, only a low percentage (<0.01%) of total GSH is in the oxidized form (GSSG), and GSSG increases under conditions of marked toxicity or oxidative stress. In these conditions, there is an increased export of GSSG from the cells, presumably through an active transport mechanism, even though most cell types contain appreciable activity of GSSG reductase. It has been suggested that active export of GSSG may be an emergency mechanism to protect cells from the toxic effects of GSSG. The exact nature of this transport system is unknown, and the question as to how much GSSG is normally transported is difficult to answer because intracellular GSSG levels are very low, and accurate measurements in the presence of large amounts of GSH is a technical problem. GSSG transport has been reported in erythrocytes, the liver and lens [69].

Uptake of intact glutathione

Since 1986, several authors, mostly from the same research group, have described uptake of intact GSH by type II pneumocytes, kidney cells and the small intestine [70–75]. The mechanism seems to be present only in cells designed to salvage GSH from the extracellular environment. All cell types are epithelial in origin and located in areas that are readily exposed to oxidative or xenobiotic injury. Uptake of intact GSH was not found in liver cells, fibroblasts and (pulmonary) endothelial cells [13]. The transporter was found to be sodium-dependent, and could be inhibited by about 60% by γ -glutamyl compounds, such as γ -glutamylglutamate, probenecid and ophthalmic acid [70, 71]. Anaerobic conditions completely inhibited the uptake, implying that the process is an active one [72]. HAGEN and co-workers [71, 72] found that the uptake rate of intact GSH was greater than the rate of intracellular synthesis in renal tubular cells; the rate of GSH formation was $0.15\text{--}0.28\text{ nmol}\cdot 10^{-6}\text{ cells}\cdot\text{min}^{-1}$ and the initial uptake rate $1.1\text{ nmol}\cdot 10^{-6}\text{ cells}\cdot\text{min}^{-1}$ at an extracellular concentration of GSH or amino acids of 1 mM. In type II cells, the uptake had the same characteristics as in kidney cells; it was a sodium-dependent active process, with uptake against a substantial concentration gradient and inhibition by γ -glutamyl compounds. The synthetic rate was again a small fraction of the transport rate [70, 73–75]. Type I cells do not have the capacity to take up intact GSH [14].

In epithelial cells, the uptake of intact GSH might, thus, play an important role in GSH turnover and protection against oxidative stress. Although all these studies present data on the time course of GSH uptake, the kinetic properties of the uptake system are not known and the transporter has not been identified. As all investigators, except JENKINSON *et al.* [75], used relatively high GSH concentrations (1 mM), passive diffusion cannot be excluded, because no data on the uptake at 4°C are presented. JENKINSON *et al.* [75] used a more physiological GSH concentration of 0.02 mM, and demonstrated an uptake of only $4.7\text{ pmol}\cdot 10^{-6}\text{ cells}$ in 5 min, which is of minor significance. In our own (unpublished) studies, it was difficult to demonstrate any uptake, and other scientists also appear to have had problems.

Possible implications for therapy

It is important to make a distinction between curative treatment with antioxidants to restore GSH levels in GSH-depleted cells, and "preventive" treatment to increase antioxidant levels in otherwise normal cells, in order to protect them against an expected attack by oxidants. In the former situation, the cells will readily take up cysteine analogues but the latter situation is likely to be more complicated, because intracellular GSH synthesis is subject to a negative feedback by GSH itself. There are, nevertheless, several possibilities to circumvent this problem, but these are dependent on the target cells.

Because epithelial cells (type II pneumocytes) may be equipped with an uptake system for intact GSH, exposure of these cells to GSH might increase intracellular levels. However, this system is not present in liver, endothelial cells and fibroblasts. The same applies for the γ -GT pathway, by which precursor amino acids, cystine and less, efficiently, cysteine, are taken up into the cell, bound to the γ -glutamyl moiety of GSH, thus forming γ -glutamyl-cyst(e)ine, and hence bypassing the rate-limiting enzyme of the GSH synthesis, γ -glutamyl-cysteine synthetase. Another possibility is the induction of the cystine transport system by oxidative stress or treatment with DEM, but it is not certain that this system is inducible in type II pneumocytes. Furthermore, there is evidence that a more oxidized environment (*i.e.* high cystine) will force the bidirectional GSH transporter, at least in liver cells, to retain GSH inside the cell.

In our *in vivo* experiments with N-acetylcysteine (NAC) and 85% hyperoxia, we demonstrated that daily *i.p.* injections of $200\text{ mg}\cdot\text{kg}^{-1}$ NAC indeed increased ELF GSH levels, but lowered type II cell GSH, and led to higher type II cell γ -GT and higher ELF γ -GT activities [68]. These findings might support the presence of a bidirectional GSH transporter, and demonstrate that a more reduced environment will lead to GSH release from the type II cell into the ELF. The question is whether these higher ELF GSH levels, achieved at the expense of lower intracellular GSH levels, are beneficial for the cell. Further investigations are needed, although we demonstrated significantly more type II cell damage in the NAC-treated animals, as indicated by 24 h thymidine incorporation. Because the different cell types exhibit important differences in the uptake mechanisms, it is probably wrong to apply a single treatment regimen for all target cells, and a more cell-specific strategy is presumably more appropriate.

Conclusion

Studying the turnover of GSH is, in fact, a study of cysteine/cystine metabolism, since GSH can be regarded as a vehicle for cysteine transport. As a free amino acid, cysteine is more efficiently transported than cystine, but in most cell types, with the probable exception of lung type II cells, cystine transport is inducible by oxidative stress or amino acid starvation. Cells of epithelial origin located in areas that are readily exposed to oxidative or xenobiotic injury, are equipped with two other uptake pathways, in addition to the amino acid uptake pathways for cysteine and cystine. The first is the uptake of intact GSH, the second the γ -glutamyl cycle,

by which cystine in particular, and, less efficiently, cysteine, and other precursor molecules for *de novo* GSH synthesis can be taken into the cell.

Recently, more information concerning the extracellular transport of cysteine has become available for liver cells. It is remarkable that the redox state of the extracellular milieu influences the bidirectional GSH transporter. A more oxidized environment, *i.e.* characterized by high cystine levels, stimulates the cell to retain GSH, whilst a more reduced state facilitates GSH efflux. Identification of a similar transport system in type II pneumocytes would probably shed new light on the origin of the increased GSH levels found in the ELF of the lung during hyperoxia and in smokers. Further insight into the mechanism by which intra- and extracellular glutathione levels are established would probably lead to new therapeutic approach to increasing (intracellular) antioxidant defence.

References

- Cantin A, North S, Hubbard R, Crystal R. Normal epithelial lining fluid contains high levels of glutathione. *J Appl Physiol* 1987; 63: 152–157.
- Jenkinson SG, Black RD, Lawrence RA. Glutathione concentrations in rat lung bronchoalveolar lavage fluid: effects of hyperoxia. *J Lab Clin Med* 1988; 112: 345–351.
- Heffner JE, Repine JE. Pulmonary strategies of antioxidant defence: state of the art. *Am Rev Respir Dis* 1989; 140: 531–554.
- Cantin AM, Hubbard RC, Crystal RG. Glutathione deficiency in the epithelial tract in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 1989; 139: 370–372.
- Roum RJ, Buhl R, McElvaney NG, *et al.* Cystic fibrosis is characterized by a marked reduction in glutathione levels in pulmonary epithelial lining fluid (Abstract). *Am Rev Respir Dis* 1990; 141: A87.
- Buhl R, Jaffe HA, Hollroyd KJ, *et al.* Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. *Lancet* 1988; 2: 1294–1298.
- Pacht ER, Timerman AP, Lykens MG, Merola AJ. Deficiency of alveolar fluid glutathione in patients with sepsis and the adult respiratory distress syndrome. *Chest* 1991; 100: 1397–1403.
- Bernard GR. N-acetylcysteine in experimental and clinical acute lung injury. *Am J Med* 1991; S3C: S54–S59.
- Crapo JD, Young SL, Fram EK, Pinkerton KE, Barry BE, Crapo R. Morphometric characteristics of cells in the alveolar region of mammalian lungs. *Am Rev Respir Dis* 1983; 128: S42–S48.
- Finkelstein JN. Physiologic and toxicologic responses of alveolar type II cells. *Toxicology* 1990; 60: 41–52.
- Dinsdale D, Green JA, Manson MM, Lee MJ. The ultrastructural immunolocalization of γ -glutamyltranspeptidase in rat lung: correlation with the histochemical demonstration of enzyme activity. *Histochem J* 1992; 24: 144–152.
- Kinnula VL, Crapo JD, Raivio KO. Biology of disease. Generation and disposal of reactive oxygen metabolites in the lung. *Lab Invest* 1995; 73: 3–19.
- Doeleman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9: 381–400.
- Deneke SM, Fanburg BL. Regulation of cellular glutathione. *Am J Physiol* 1989; 257: L163–L173.
- Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 1983; 52: 711–760.
- Reed DJ, Fariss MW. Glutathione depletion and susceptibility. *Pharmacol Rev* 1984; 36 (Suppl. 2): S25–S33.
- Morris PE, Bernard CR. Significance of glutathione in lung disease and implications for therapy. *Am J Med Sci* 1994; 307:119–127.
- Meister A, Tate SS. Glutathione and related γ -glutamyl compounds: biosynthesis and utilization. *Annu Rev Biochem* 1976; 45: 559–604.
- Burgunder JM, Lauterburg BH. Decreased production of glutathione in patients with cirrhosis. *Eur J Clin Invest* 1987; 17: 408–414.
- Wendel A, Cikryt P. The level and half-life of glutathione in human plasma. *FEBS Lett* 1980; 120: 209–211.
- Meister A. Methods for the selective modification of glutathione metabolism and study of glutathione transport. *Methods Enzymol* 1985; 113: 571–586.
- Bannai S. Transport of cystine and cysteine in mammalian cells. *Biochem Biophys Acta* 1984; 779: 289–306.
- Deneke SM, Steiger V, Fanburg B. Effect of hyperoxia on glutathione levels and glutamic acid uptake in endothelial cells. *J Appl Physiol* 1987; 63: 1966–1971.
- Deneke SM, Lawrence RA, Jenkins SG. Endothelial cell cystine uptake and glutathione increase after N,N-bis(2-chloroethyl)-N-nitrosourea exposure. *Am J Physiol* 1992; 262: L301–L304.
- Bannai S, Tateishi N. Role of membrane transport in metabolism and function of glutathione in mammals. *J Membr Biol* 1986; 89: 1–8.
- Bannai S. Induction of cystine and glutamate transport activity in human fibroblasts by diethyl maleate and other electrophilic agents. *J Biol Chem* 1984; 259: 2435–2440.
- Bannai S, Sato H, Ishii T, Sugita T. Induction of cystine transport activity in human fibroblasts by oxygen. *J Biol Chem* 1989; 255: 2372–2376.
- Bannai S, Sato H, Ishii T, Taketani S. Enhancement of glutathione levels in mouse peritoneal macrophages by sodium arsenite, cadmium chloride and glucose/glucose oxidase. *Biochem Biophys Acta* 1991;1092: 175–179.
- Deneke S. Induction of cystine transport in bovine pulmonary artery endothelial cells by sodium arsenite. *Biochem Biophys Acta* 1992; 1109: 127–131.
- Deneke SM, Baxter DH, Phelps D, Fanburg B. Increase in endothelial cell glutathione and precursor amino acid uptake by diethyl maleate and hyperoxia. *Am J Physiol* 1989; 257: L265–L271.
- Phelps D, Deneke S, Daley D, Fanburg B. Elevation of glutathione levels in bovine pulmonary endothelial cells by N-acetyl cysteine. *Am J Respir Cell Mol Biol* 1992; 713: 293–298.
- Christensen H. Exploiting amino acid structure to learn about membrane transport. *Adv Enzymol* 1979; 49: 41–100.
- Bukowski DM, Deneke SM, Lawrence RA, Jenkinson SG. A noninducible cystine transport system in rat alveolar type II cells. *Am J Physiol* 1995; 268: L21–L26.
- Phelps DT, Deneke SM, Baxter DF, Fanburg B. Erythrocytes fail to induce glutathione in response to diethyl maleate or hyperoxia. *Am J Physiol* 1989; 257: L272–L276.
- Bannai S, Ishii T. Transport of cystine and cysteine and cell growth in cultured human diploid fibroblasts: effect of glutamate and homocysteate. *J Cell Physiol* 1982; 112: 265–272.
- Bannai S, Kitamura E. Transport interaction of L-cystine and L-glutamine in human diploid fibroblasts in culture. *J Biol Chem* 1980; 255: 2372–2376.
- Watanabe H, Bannai S. Induction of cystine transport activity in isolated rat hepatocytes by sulfobromophthalein and other electrophilic agents. *Hepatology* 1986; 6: 1361–1368.
- Watanabe H, Bannai S. Induction of cystine transport

- activity in mouse peritoneal macrophages. *J Exp Med* 1987; 165: 628–640.
39. Miura K, Ishii T, Sugita Y, Bannai S. Cystine uptake and glutathione level in endothelial cells exposed to oxidant stress. *Am J Physiol* 1992; 262: C50–C58.
 40. Cotgreave IA, Schuppe-Koistinen I. A role for γ -glutamyl transpeptidase in the transport of cystine into human endothelial cells: relationship to intracellular glutathione. *Biochim Biophys Acta* 1992; 1222: 375–382.
 41. Deneke SM, Susanto I, Vogel KA, Williams CE, Lawrence RA. Mechanism of use of extracellular glutathione by lung epithelial cells and pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* 1995; 12: 662–668.
 42. Cotgreave IA, Constantin D, Moldéus P. Nonxenobiotic manipulation and sulfur precursor specificity of human endothelial cell glutathione. *J Appl Physiol* 1991; 70: 1220–1227.
 43. van Klaveren RJ, Hoet PHM, Pype J, Demedts M, Nemery B. Increase in γ -glutamyltransferase by glutathione depletion in rat type II pneumocytes. *Free Rad Biol Med* 1997; 22: 525–534.
 44. Meister A, Tate SS, Griffith OW. γ -Glutamyl transpeptidase. *Methods Enzymol* 1981; 77: 237–253.
 45. Griffith OW, Meister A. Translocation of intracellular glutathione to membrane-bound γ -glutamyl transpeptidase as a discrete step in the γ -glutamyl cycle: glutathionuria after inhibition of transpeptidase. *Proc Natl Acad Sci USA* 1979; 76: 268–272.
 46. Griffith OW, Bridges RJ, Meister A. Transport of γ -glutamyl amino acids: role of glutathione and γ -glutamyltranspeptidase. *Proc Natl Acad Sci USA* 1979; 76: 6319–6322.
 47. Curthoys NP, Huhey RP. Characterization and physiological function of rat renal gamma-glutamyl transpeptidase. *Enzyme* 1979; 24: 383–403.
 48. Ingbar HI, Hepler K, Dowin R, et al. γ -Glutamyl transpeptidase is a polarized alveolar epithelial membrane protein. *Am J Physiol* 1995; 269: L261–L271.
 49. Ingbar DH, Dowin R, Jamieson JD. γ -Glutamyltransferase as a quantitative membrane protein marker of type I pneumocytes in rat lung. *Am Rev Respir Dis* 1989; 139: A256.
 50. Joyce-Brady M, Takahashi Y, Oakes SM, et al. Synthesis and release of amphipathic γ -glutamyltransferase by the pulmonary alveolar type 2 cell. *J Biol Chem* 1994; 269: 14219–14226.
 51. Forman HJ, Skelton DT. Protection of alveolar macrophages from hyperoxia by γ -glutamyl transpeptidase. *Am J Physiol* 1990; 259: L102–L107.
 52. Nielson DW, Goerke J, Clements JA. Alveolar subphase pH in the lungs of anesthetized rabbits. *Proc Natl Acad Sci USA* 1981; 78: 7119–7123.
 53. Funkhouser JD, Tangada SD, Peterson RDA. Ectopeptidases of alveolar epithelium: candidates for roles in alveolar regulating mechanisms. *Am J Physiol* 1991; 260: L381–L385.
 54. Morris HR, Taylor GW, Jones CM, Piper PJ, Samhoun MN, Tippins JR. Slow-reacting substances (leukotrienes): enzymes involved in their biosynthesis. *Proc Natl Acad Sci USA* 1992; 79: 4838–4842.
 55. Tate SS, Meister A. γ -Glutamyl transpeptidase from kidney. *Methods Enzymol* 1995; 113: 400–419.
 56. Shi MI, Gozal E, Choy HA, Forman HJ. Extracellular glutathione and γ -glutamyl-transpeptidase prevent H₂O₂-induced injury by 2,3-dimethoxy-1,4-naphtho-quinone. *Free Rad Biol Med* 1993; 15: 57–67.
 57. Rajpert-De Meyts E, Shi M, Chang M, et al. Transfection with γ -glutamyl transpeptidase enhances recovery from glutathione depletion using extracellular glutathione. *Toxicol Appl Pharmacol* 1992; 114: 56–62.
 58. Kugelman A, Choy HA, Liu R, Shi MM, Gozal E, Forman HJ. γ -Glutamyl-transpeptidase is increased by oxidative stress in rat alveolar L2 epithelial cells. *Am J Respir Cell Mol Biol* 1994; 11: 586–592.
 59. Edwards AM, Lucs CS. Induction of γ -glutamyltransferase in primary cultures of normal rat hepatocytes by liver tumor promoters and structurally related compounds. *Carcinogenesis* 1985; 6: 733–739.
 60. Meister A. Glutathione metabolism and its selective modification. *J Biol Chem* 1988; 263: 17205–17208.
 61. Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (s-n-butylhomocysteine sulfoximine). *J Biol Chem* 1979; 254: 7558–7560.
 62. Kang YJ, Feng YI, Hachter EL. Glutathione stimulates A549 cell proliferation in glutamine-deficient culture: the effect of glutamate supplementation. *J Cell Physiol* 1994; 161: 589–596.
 63. Lu SC, Kuhlenkamp J, Ge J, Sun W, Kaplowitz N. Specificity and directionality of thiol effects on sinusoidal glutathione transport in rat liver. *Mol Pharmacol* 1994; 46: 578–585.
 64. Lu SC, Ge J, Huang H, Kuhlenkamp J, Kaplowitz N. Thiol-disulfide effects on hepatic glutathione transport. Studies in cultured rat hepatocytes and perfused livers. *J Clin Invest* 1993; 92: 1188–1197.
 65. Garcia-Ruiz C, Fernandez-Checa JC, Kaplowitz N. Bidirectional plasma membrane transport of reduced glutathione in intact hepatocytes and membrane vesicles. *J Biol Chem* 1992; 267: 22256–22264.
 66. Sies H, Graf P. Hepatic thiol and glutathione efflux under the influence of vasopressin, phenylephrine and adrenaline. *Biochem J* 1985; 226: 545–549.
 67. Smith CV, Jones DP, Guenther TM, Lash LH, Lauterburg BH. Compartmentalization of glutathione: implications for the study of toxicity and disease. *Toxicol Appl Pharmacol* 1996; 140: 1–12.
 68. van Klaveren RJ, Demedts M, Nemery B. Decreased γ -glutamyltransferase activity in isolated rat type II pneumocytes after *in vivo* exposure to hyperoxia. *Am J Respir Crit Care Med* 1996; 153: A851.
 69. Sies H, Akerboom ThPM. Glutathione disulfide (GSSG) efflux from cells and tissues. *Methods Enzymol* 1984; 105: 445–451.
 70. Hagen TM, Brown LA, Jones DP. Protection against paraquat-induced injury by exogenous GSH in pulmonary alveolar type II cells. *Biochem Pharmacol* 1986; 35: 4537–4542.
 71. Hagen TM, Jones DP. Transepithelial transport of glutathione in vascularly perfused small intestine of rat. *Am J Physiol* 1987; 252: G607–G613.
 72. Hagen TM, Aw TY, Jones DP. Glutathione uptake and protection against oxidative injury in isolated kidney cells. *Kidney Intern* 1988; 34: 74–81.
 73. Brown LAS, Bai C, Jones DP. Glutathione protection in alveolar type II cells from fetal and neonatal rabbits. *Am J Physiol* 1992; 262: L305–L312.
 74. Bai C, Brown LAS, Jones DP. Glutathione transport by type II cells in perfused rat lung. *Am J Physiol* 1994; 267: L447–L455.
 75. Jenkinson SG, Lawrence RA, Zamora CA, Deneke SM. Induction of intracellular glutathione in alveolar type II pneumocytes following BCNU exposure. *Am J Physiol* 1994; 266: L125–L130.