

Short communication

Vitamin C supply to bronchial epithelial cells linked to glutathione availability in elf — A role for secreted γ -glutamyltransferase?

Alessandro Corti, Maria Franzini, Alessandro F. Casini, Aldo Paolicchi, Alfonso Pompella *

Dipartimento di Patologia Sperimentale, Università di Pisa, Scuola Medica — Pisa, Italy

Received 1 February 2007; received in revised form 15 May 2007; accepted 29 June 2007
Available online 29 January 2008

Abstract

Previous work in our laboratory has shown that glutathione (GSH) availability is linked to cellular supply of ascorbic acid, through the action of γ -glutamyltransferase (GGT). This enzyme activity is expressed in bronchial epithelia, and is also secreted in epithelial lining fluid. We verified thus the possibility that GGT-mediated metabolism of glutathione may favour the supply of ascorbic acid to bronchial cells. Using human BEAS-2B cells transfected with GGT cDNA, as well as WT cells exposed to ELF-like GGT concentrations, we observed that indeed much higher (5–10 fold) levels of ascorbic acid are accumulated in the presence of GSH and active GGT. The data suggest that administration of aerosolized GSH to CF patients may as well concur to sustain the vitamin C status of bronchial epithelia.

© 2007 Published by Elsevier B.V. on behalf of European Cystic Fibrosis Society.

Keywords: Airway inflammation; Oxidative stress; Epithelial lining fluid; Vitamin C supply; Glutathione; Gamma-glutamyltransferase

1. Introduction

The production of oxidative damage to cells and tissues during chronic lung inflammation is a well established observation, e.g. in cystic fibrosis (CF), [1]. Accumulation of neutrophils causes continued production of reactive oxygen species. Lipoperoxidation is indicated by elevated concentrations of plasma malondialdehyde, breath pentane and ethane and plasma hydroperoxides, as well as by depletion of major lipoperoxidation substrates, such as linoleic and arachidonic acids. Increased oxidative damage to DNA was shown by elevated urinary concentrations of 8-hydroxyguanosine. Moreover, in stable CF patients elevated concentrations were described of isoprostanes [2]. Consequence of continued oxidative stress is progressive lung damage and pulmonary fibrosis, being responsible for over 90% of the observed mortality. In particular, in CF patients the problem

is exacerbated by a deficiency in systemic antioxidant activity partly due to malabsorption secondary to pancreatic insufficiency, and antioxidant supplementation is therefore recommended [2].

The importance of adequate levels of *extracellular* antioxidants, i.e. those present in the lung epithelial lining fluid (ELF), has been equally stressed. The major antioxidant glutathione (GSH) is present at high concentrations in ELF, where it can serve as a direct scavenger of prooxidant agents produced during inflammation. Ascorbic acid (Vit. C) is another important antioxidant in ELF [3], and its protective effect against oxidative injury has been documented in type II cells [4]. ELF compartment also contains significant levels of γ -glutamyltransferase (GGT), but the function played by this enzyme activity in pulmonary fluids remains to be determined. Previous studies in our laboratory have shown that GGT activity can give rise to prooxidant reactions with a series of effects on cellular pathophysiology [5]), including the oxidation of Vit. C to dehydroascorbic acid (DHA) [6]. Since – at variance with the reduced vitamin – DHA is efficiently transported through GLUT transporters of the cell membrane and is then reduced back to Vit. C by cellular

* Corresponding author. Department of Experimental Pathology, University of Pisa Medical School, Via Roma 55 - 56126 Pisa, Italy.

E-mail address: apompella@biomedunipi.it (A. Pompella).

reductases, the possibility exists that GGT-mediated oxidation of Vit. C in ELF may facilitate cellular transport of vitamin C.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all reagents were from Sigma Chemical Co. (Milan, Italy).

2.2. Cell culture and treatments

Human bronchial epithelial BEAS-2B cells were stable transfected with a vector (pcDNA; Invitrogen) containing the full-length cDNA of human GGT. A lipid transfection according to Lipotaxi[®] mammalian transfection kit protocol (Stratagene) was used and stable transfected clones, carrying resistance to the antibiotic geneticin (G418), were selected. At the end of procedures one clone with high GGT activity (clone “E1”) and another, transfected with the empty vector (clone “N7”) were selected and used for the subsequent experiments. Transfected cells were routinely grown in BEGM[®] medium (Cambrex, MD), supplemented with 2 mM L-glutamine (L-Gln) and 0.2 µg/mL G418 (Invitrogen/Gibco, Milan, Italy), at 37 °C in a 5%/95% CO₂/air atmosphere. Wild type cells were grown in the same medium in the absence of G418.

For treatments, culture medium was replaced with Hank’s buffer, pH 7.4, containing ascorbic acid (60 µmol/L), GSH (400 µmol/L), glycyl-glycine (4 mmol/L) and ADP-chelated FeCl₃ (100/10 µmol/L, respectively). Where indicated, soluble GGT (100 mU/mL) was added to incubation mixtures. Vit. C concentrations were determined by high-pressure liquid chromatography (HPLC) as described previously [6].

2.3. Other determinations

Determination of low molecular weight thiols was carried out as described previously [7]. Briefly, cells were lysed in TCA 5%, intracellular thiols were treated with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F; Fluka) and their concentration was determined by a high-pressure liquid chromatography system equipped with a C-18 reverse-phase column (Resolve; Waters, USA) and a fluorimetric detector (RF-551;

Table 1
GGT expression vs. intracellular levels of glutathione and cysteine after GGT-cDNA transfection of BEAS-2B cells (“E1” cells)

Cells	GGT activity (U/mg cell protein)	Intracellular GSH (nmol/mg cell protein)	Intracellular cysteine (nmol/mg cell protein)
WT	(traces)	17.1±1.7	3.3±1.1
E1 (GGT-cDNA)	4.1±0.2	9.4±1.9 (*)	1.7±0.3 (**)

Values reported are means±SD of 3 independent determinations. (*) $P < .001$. (**) $P < .01$.

Table 2
Progressive release of active GGT enzyme protein in culture medium by GGT-transfected BEAS-2B cells

Cells	GGT activity (mU/mg cell protein)	
	48 h culture	72 h culture
WT	ND	0.6±0.1
E1 (GGT-cDNA)	305.0±15.06	368.1±6.30

Values reported are means±SD of 3 independent determinations. ND, not detectable.

Shimadzu, Japan). GSSG determinations were performed as described previously [17].

For preparation of cell membrane and cytosolic fractions, cell monolayers were collected in hypotonic lysis buffer (10 mmol/L Tris-HCl, pH 7.8), and cells were disrupted by a tight-fitting glass-glass Dounce homogenizer (20 strokes). Crude homogenates were centrifuged at 400 g (10 min, RT) and then 100,000 g (60 min, 4 °C). The resulting supernatants (cytosol) were collected, and the pellets (cell membranes) were resuspended in hypotonic buffer. Both samples were stored at -20 °C until GGT activity determinations.

GGT activity was determined spectrophotometrically using γ -glutamyl-*p*-nitroanilide as substrate, as previously described [5]. Incubation media were centrifuged at 10,000 g (10 min, 4 °C) before GGT determinations.

Protein content was determined by the method of Bradford (Bio-Rad[®] protein assay).

Statistical analysis of data was done using ANOVA with Neuman-Keuls test for multiple comparisons.

3. Results

As compared to wild type cells, the GGT-transfected E1 clone presented with a high GGT activity (4.1±0.2 U/mg cell protein), while both WT and empty vector-transfected N7 cells showed only traces of enzyme activity (Table 1). When the enzyme activity of E1 cells was separately determined, 2–3% of total expressed GGT activity was found in the cytosolic fraction (data not shown). GGT-transfected cells presented with a significant decrease (approx. 50%) in the intracellular levels of both GSH and its precursor cysteine, while no significant changes were appreciable in WT or N7 cells. The GSH decrease was not accounted for by oxidation to GSSG, whose intracellular levels were not different in E1 as compared to WT cells (data not shown). E1 cells also acquired the ability to release enzymatically active GGT protein in the culture medium (Table 2); a substantial level of soluble enzyme was detectable in medium at 48 h of culture, and further increased with culture time.

Fig. 1 reports data concerning metabolism of extracellular GSH and oxidation of Vit. C. GGT-transfected E1 cells were capable of oxidizing Vit. C to DHA (Fig. 1B) while metabolizing extracellular GSH (Fig. 1A), and this was accompanied by a marked intracellular uptake of ascorbic acid (Fig. 1E). No such effects were observed in WT cells (Fig. 1C–D–E). In additional experiments, WT and sham-

transfected N7 cells were exposed to soluble GGT, added exogenously at concentrations comparable with those found in epithelial lining fluid (ELF) [8–10]. As expected, presence of GGT activity in the medium caused GSH consumption and Vit. C oxidation (*not shown*), as well as marked facilitation of Vit. C uptake in WT and N7 cells as well (Fig. 1E).

4. Discussion

The role played by γ -glutamyltransferase in airway epithelia and epithelial lining fluids is not fully understood yet. GGT catalyzes the first step in the degradation of extracellular glutathione, i.e. the hydrolysis of the gamma-

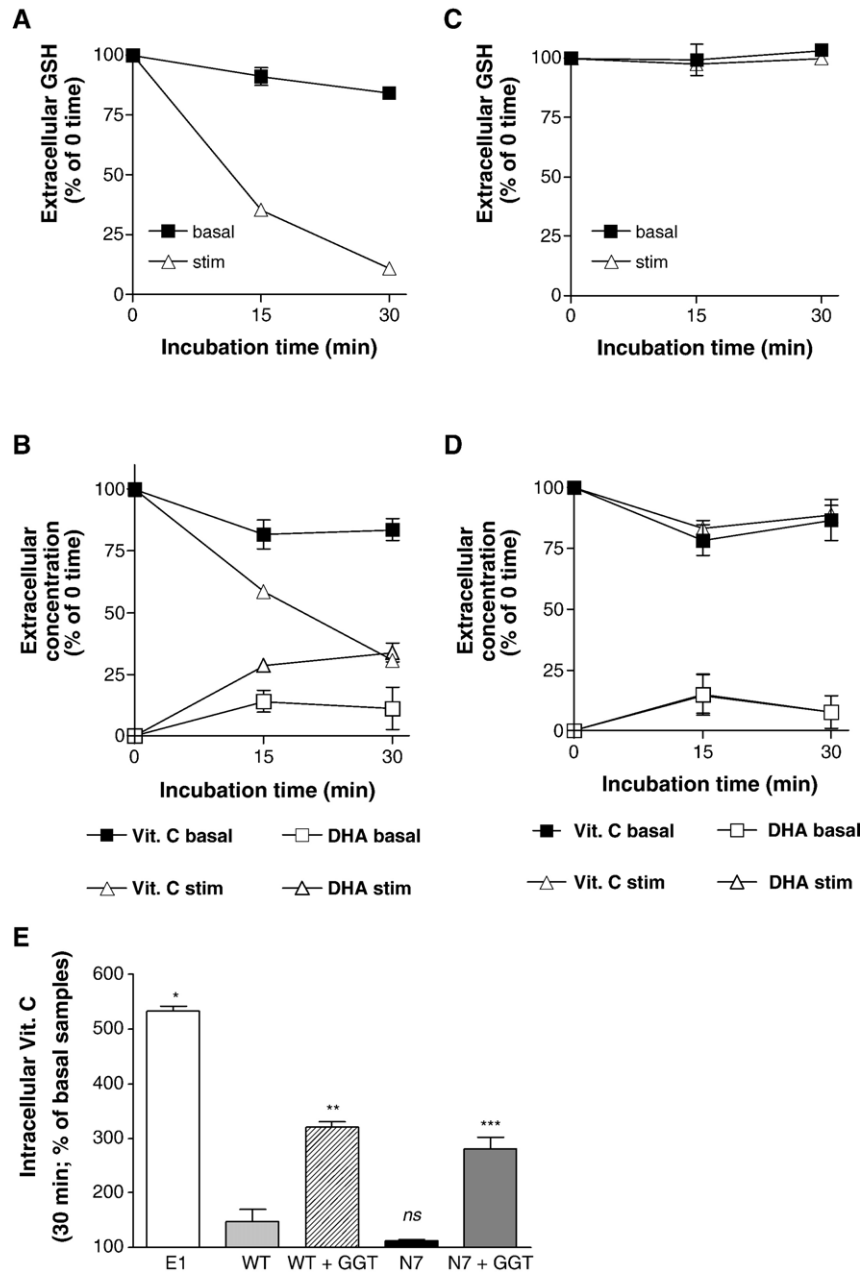


Fig. 1. GGT activity facilitates cellular uptake of ascorbic acid in human bronchial BEAS-2B cells. *Panel A*: metabolism of extracellular GSH by GGT-transfected E1 cells, in basal conditions or conditions for “stimulated” GGT activity (*stim*). *Panel B*: oxidation of ascorbic acid (*Vit. C*) to dehydroascorbic acid (*DHA*) by E1 cells in the same incubations. GSH was added at final conc. of 400 μ M. “Stimulation” of GGT was achieved by addition of glycyl–glycine (as acceptor for transpeptidation reaction; 4 mM). Incubation mixtures also contained ascorbic acid (60 μ M) and ADP-chelated FeCl_3 (100–10 μ M, respectively) (6). *Panels C, D*: WT cells cannot consume extracellular GSH, nor oxidize *Vit. C* to *DHA*. *Panel E*: intracellular accumulation of ascorbic acid after 30 min of incubation. E1 cells achieved ascorbic acid levels approx. 10-fold higher than WT cells. Addition of soluble GGT at ELF-like concentration (100 mU/mL) (8) likewise produced a marked increase of *Vit. C* (5-fold) in WT and sham-transfected N7 cells as well. Data are means \pm SD of three separate experiments. (*) $P < 0.001$ as compared to WT cells and N7 cells; (**) $P < 0.001$ as compared to WT cells; (***) $P < 0.001$ as compared to N7 cells; *ns*, not significantly different from WT.

glutamyl bond between glutamate and cysteine. In this process GGT releases cysteinyl–glycine, which is subsequently cleaved to cysteine and glycine by plasma membrane dipeptidase activities. Thus, GGT-mediated metabolism of extracellular GSH provides cells with a means for the recovery of cysteine, necessary for the intracellular resynthesis of GSH. In this perspective, GGT can be seen as an important factor in the maintenance of adequate antioxidant defences within the cell; this likely explains why exposure to prooxidant agents can induce the expression of GGT, as shown e.g. in lung epithelial cells after challenge with a sublethal oxidative stress [11]. Several studies have shown however the ability of GGT to play a *prooxidant* role under selected conditions, eventually leading to production of reactive oxygen species (superoxide anion, hydrogen peroxide), capable of stimulating prooxidant reactions. We have previously characterized the details of biochemical processes involved (reviewed in [12]), and described some of the redox effects occurring at the cellular level, e.g. on activation of transcription factors [13,14]. Data reported in the present study confirm previous observations on GGT-dependent prooxidant reactions. Intracellular GSH decrease, such as reported in Table 1, is a known phenomenon in GGT-transfected cells [15–17], and is likely the result of GSH consumption caused by the low-level oxidative stress imposed to cells by GGT activity itself [18]. The same explanation can be invoked to explain the observed lower levels of cysteine in E1 cells: extracellular reactions promoted by GGT can favour the oxidation of cysteine to cystine, whose cellular uptake is less efficient than that of cysteine [19]. A minor percentage of transfected GGT activity was detectable in the cytosolic fraction of E1 cells, which raises the possibility that the observed GSH decrease may actually result from GGT-mediated metabolism in the cytosol. On the other hand, the possibility cannot be excluded that some degree of contamination of cytosol with GGT released from the cell membrane fraction may have occurred during sample preparation procedures.

As an effect of oxidative processes mediated by GGT activity in the extracellular space extracellular ascorbic acid was oxidized by E1 bronchial cells to DHA, which resulted in a favouring effect on cellular Vit. C status. The same phenomenon was previously observed and characterized in melanoma cells [6], and possibly represents an additional, as yet unexplored role of GGT in extracellular spaces. GGT is induced by oxidative stress in a wide range of cell types, including type 2 pneumocytes [7]. It was reported that GGT protein is released in ELF in conjunction with phosphatidylcholine of the surfactant [20], but the mechanisms underlying such phenomenon remain unclear. As reported in Table 2, GGT-transfected BEAS-2B cells become capable of releasing GGT in the medium, and may thus represent a suitable model to investigate this aspect. Interestingly, GGT ELF concentrations were found to increase several fold in CF patients, even if the mechanism underlying such increase was not determined [21]. Increased expression and secretion

of GGT was generally interpreted in connection with its role in metabolism of GSH and cellular thiol supply, as discussed above. Our data in addition suggest that GGT secreted in ELF may contribute to the maintenance of ascorbate-based antioxidant defences in bronchial epithelial cells. From levels of ≈ 20 mU/mL detectable in ELF of healthy subjects, GGT activity is known to increase up to >100 mU/mL during lung inflammation in CF patients (21). Indeed, our data indicate that when exposed to exogenous GGT in the same concentration range BEAS-2B cells do increase their uptake of Vit. C (Fig. 1E).

On the other hand, the effect can take place provided that sufficient levels of GSH are available in ELF, such to sustain enzyme activity. CF patients actually present with *decreased* levels of glutathione, likely due to increased consumption during inflammation-related oxidative stress [22]. Moreover, it has been proposed that GSH deficiency in CF ELF may derive – at least in part – from the same alterations in CFTR channel which originate the disease, leading to abnormal GSH transport across the membranes of epithelial cells [9,23]. CF patients also present with decreased levels of ascorbic acid [24]. Altogether, regardless the cause underlying depletion of ELF GSH in CF patients, it is conceivable that current attempts to reconstitute such levels by administration of aerosolized buffered GSH may also produce indirect, beneficial effects on the ascorbic acid status of airway epithelia, contributing to reinforcement of cellular antioxidant defences.

Acknowledgment

The present study was supported by a grant from *Fondazione per la Ricerca sulla Fibrosi Cistica-Onlus* (Verona, Italy).

References

- [1] McGrath LT, Mallon P, Dowey L, et al. Oxidative stress during acute respiratory exacerbations in cystic fibrosis. *Thorax* 1999;54:518–23.
- [2] Wood LG, Fitzgerald DA, Lee AK, et al. Improved antioxidant and fatty acid status of patients with cystic fibrosis after antioxidant supplementation is linked to improved lung function. *Am J Clin Nutr* 2003;77:150–9.
- [3] Cross CE, van der Vliet A, O'Neill CA, et al. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994;102:185–91.
- [4] Brown LA, Harris FL, Jones DP. Ascorbate deficiency and oxidative stress in the alveolar type II cell. *Am J Physiol* 1997;273:L782–8.
- [5] Dominici S, Valentini M, Maellaro E, et al. Redox modulation of cell surface protein thiols in U937 lymphoma cells: the role of gamma-glutamyl transpeptidase-dependent H2O2 production and S-thiolation. *Free Radic Biol Med* 1999;27:623–35.
- [6] Corti A, Raggi C, Franzini M, et al. Plasma membrane gamma-glutamyltransferase activity facilitates the uptake of vitamin C in melanoma cells. *Free Radic Biol Med* 2004;37:1906–15.
- [7] Corti A, Paolicchi A, Franzini M, et al. The S-thiolating activity of membrane γ -glutamyltransferase: formation of cysteinyl-glycine mixed disulfides with cellular proteins and in the cell microenvironment. *Antioxid Redox Signal* 2005;7:911–8.
- [8] Van Klaveren RJ, Hoet PH, Pype JL, et al. Increase in gamma-glutamyltransferase by glutathione depletion in rat type II pneumocytes. *Free Radic Biol Med* 1997;22:525–34.

- [9] Gao L, Kim KJ, Yankaskas JR, et al. Abnormal glutathione transport in cystic fibrosis airway epithelia. *Am J Physiol* 1999;277:L113–8.
- [10] Stites SW, Plautz MW, Bailey K, et al. Increased concentrations of iron and isoferitins in the lower respiratory tract of patients with stable cystic fibrosis. *Am J Respir Crit Care Med* 1999;160:796–801.
- [11] Choi J, Liu RM, Forman HJ. Adaptation to oxidative stress: quinone-mediated protection of signaling in rat lung epithelial L2 cells. *Biochem Pharmacol* 1997;53:987–93.
- [12] Dominici S, Paolicchi A, Corti A, et al. Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyltransferase activity. *Methods Enzymol* 2005;401:483–500.
- [13] Del Bello B, Paolicchi A, Comporti M, et al. Hydrogen peroxide produced during γ -glutamyl transpeptidase activity is involved in prevention of apoptosis and maintenance of proliferation in U937 cells. *FASEB J* 1999;13:69–79.
- [14] Paolicchi A, Dominici S, Pieri L, et al. Glutathione catabolism as a signalling mechanism. *Biochem Pharmacol* 2002;64:1029–37.
- [15] Hanigan MH, Gallagher BC, Townsend DM, et al. Gamma-glutamyl transpeptidase accelerates tumor growth and increases the resistance of tumors to cisplatin in vivo. *Carcinogenesis* 1999;20:553–9.
- [16] Karp DR, Shimooku K, Lipsky PE. Expression of gamma-glutamyl transpeptidase protects ramos B cells from oxidation-induced cell death. *J Biol Chem* 2001;276:3798–804.
- [17] Franzini M, Corti A, Lorenzini E, et al. Modulation of cell growth and cisplatin sensitivity by membrane γ -glutamyltransferase in melanoma cells. *Eur J Cancer* 2006;42:2623–30.
- [18] Pompella A, De Tata V, Paolicchi A, et al. Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochem Pharmacol* 2006;71:231–8.
- [19] Knickelbein RG, Seres T, Lam G, et al. Characterization of multiple cysteine and cystine transporters in rat alveolar type II cells. *Am J Physiol* 1997;273:L1147–55.
- [20] Joyce-Brady M, Takahashi Y, Oakes SM, et al. Synthesis and release of amphipathic glutamyl transferase from the pulmonary alveolar type 2 cell. *J Biol Chem* 1994;269:14219–26.
- [21] Hull J, Vervaart P, Grimwood K, et al. Pulmonary oxidative stress response in young children with cystic fibrosis. *Thorax* 1997;52:557–60.
- [22] Roum JH, Buhl R, McElvaney N, et al. Systemic deficiency of glutathione in cystic fibrosis. *J Appl Physiol* 1993;75:2419–24.
- [23] Hudson VM. New insights into the pathogenesis of cystic fibrosis: pivotal role of glutathione system dysfunction and implications for therapy. *Treat Respir Med* 2004;3:353–63.
- [24] Back EI, Frindt C, Nohr D, et al. Antioxidant deficiency in cystic fibrosis: when is the right time to take action. *Am J Clin Nutr* 2004;80:374–84.