Role of glutathione on cell adhesion and volume.

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Abstract

Glutathione is the most abundant thiol in animal cells. Reduced glutathione (GSH) is a major intracellular antioxidant neutralizing free radicals and detoxifying electrophiles. It plays important roles in many cellular processes, including cell differentiation, proliferation, and apoptosis. In the present study we demonstrate that extracellular concentration of reduced glutathione markedly increases cell volume within few hours, in a dose-response manner. Pre-incubation of cells with BSO, the inhibitor of 7-glutamylcysteine synthetase, responsible for the first step in intracellular glutathione synthesis did not change the effect of reduced glutathione on cell volume suggesting a mechanism limited to the interaction of extracellular reduced glutathione on cell membrane. Results show that reduced GSH decreases cell adhesion resulting in an increased cell volume. Since many cell types are able to transport of GSH out, the present results suggest that this could be a fundamental self-regulation of cell volume, giving the cells a self-control on their adhesion proteins.

Keywords: glutathione, cell volume, adhesion, real-time cell analysis (RTCA), Holomonitor, A549.

INTRODUCTION

Oxidative stress results from an imbalance between reactive oxygen species production (ROS) and the abilities of biological systems to eliminate them. In that context, reduced glutathione (GSH) is the central redox agent in most aerobic organisms and a fundamental player in the fine regulation of oxidative stress. GSH is involved in numerous vital functions such as detoxifying electrophiles, scavenging free radicals, maintaining the thiol status of proteins, providing cysteine, modulating DNA synthesis, microtubular-related processes, nitric oxide homeostasis, the activity of neuromodulators as well as immune functions (reviewed in Lu 2009). As a result, reduced GSH plays important roles in cell metabolism, differentiation, proliferation and apoptosis. Disturbances in GSH homeostasis are implicated in numerous human diseases, including cancer, pathologies associated to aging, cardiovascular, inflammatory, immune degenerative diseases (Ballaroti et al. 2009) and even viral infection (Fraternale et al. 2009, Khanfar and Qaroot 2020).

Although GSH is present in the extracellular fluid, it is believed that it cannot be directly uptaken by cells. It is degraded by cells expressing the ectoprotein gammaglutamyl transpeptidase to produce dipeptides and free amino-acids uptaken by cells and used for *de novo* GSH synthesis. GSH is a tripeptide (γ-L-glutamyl-L-cysteinylglycine) synthesized by two cytosolic ATP dependent enzymes: glutamate cysteine ligase (GCL) leading to the production of γ-glutamyl-cysteine from glutamate and cysteine and glutathione synthetase (GS) involved in the ligation of γ-glutamyl-cysteine to glycine to produce γ-glutamylcysteinylglycine. After its synthesis, almost 90 % is retained into the cytoplasm (Meredith and Reed 1982, Hwang et al. 1992 in Lu 2009).

Despite the numerous effects described of reduced glutathione on cells, its influence on cell volume has never been reported so far. The present study relates experimental results pointing out the effect of reduced glutathione on cell volume on several cell lines, using different methods. Implications of such a new effect of reduced glutathione will be discussed in the light of recent data from the literature.

Material and Methods

1. Cell culture

Human lung carcinoma A549 cells, mouse fibroblasts 3T3-L1, human lung adenocarcinoma cell line NCl-H441 (ATCC, LGC Standards S.a.r.l, Molsheim, France) and 13s24 rat olfactory neuroblasts (Coronas et al, 1997) were grown in Dulbecco's Modified Eagle's Medium with low glucose (1 g/L) containing 10% fetal calf serum (DMEM 10% FCS, PAA Laboratories, Canada), streptomycin plus penicillin (100 units/mL; Sigma Aldrich, St Quentin-Fallavier, France). Before experiment, cells were dissociated by trypsin (0.05%, Sigma Aldrich) and cell concentration measured using a Sceptor pipette (Millipore, Burlington, USA). Cells were seeded at 5000 cells/cm^2 in either E-96 plates or in 96 well plates.

2. Chemicals

Chemicals: reduced glutathione, buthionine sulfoximine (BSO) and TRITC-phalloïdin were purchased from Sigma-Aldrich. BSO is an effective and specific inhibitor of 7-glutamylcysteine synthetase, the enzyme of the first step in glutathione synthesis (Griffith and Meister, 1979). Zombie Red was purchased from Ozyme (Saint-Cyr L'Ecole, France).

3. Measurements of cytotoxicity

3.1 Real-time cell analysis of impedance.

Real-time cell analysis xCELLigence biosensor (RTCA, Agilent, Santa Clara, USA) measures cell surface occupancy (i.e. by impedance measurement), expressed as cell index. It takes into account cell number, cell size and adhesion force. Cells were grown until cell index reached around one (i.e. linear proliferative phase), which may take between 48 to 72 hours. Then culture medium was replaced by a fresh one containing either reduced glutathione and/or

different inhibitors. The impedance was recorded every 15 min in a standard 37°C cell culture incubator with 5% CO_2.

3.2 Quantitative phase imaging.

Quantitative phase imaging was performed using the Holomonitor M4 digital holographic cytometer (DHC) from Phase Holographic Imaging (PHI, Lund, Sweden). The microscope was housed in a standard 37°C cell culture incubator with 5% CO_2. Cell area, optical thickness max and optical volume were measured in real-time every five minutes during ten hours after GSH addition.

3.2 Cytometry

For cell toxicity assay, at the end of experiments, living cells were incubated during 15 min. at 37°C in the presence of Zombie Red (1/1000) according to the manufacturer's procedure (Ozyme) then trypsinized and fixed in PBS with formalin 3% (Sigma Aldrich). For F-actin quantification, at the end of experiment, cells were trypsinized than fixed with PBS/ formalin 3% then incubated with PBS/ Triton 0.1%/ TRITC-phalloïdin 1 µg/mL (Sigma Aldrich) during 5 min. Cell count and fluorescence analyses were performed onto a Novocyte cytometer (ACEA) on at least 4 replicates.

4. Statistical analysis

Data presented are representative experiments performed at least in triplicates, as mean values ± SEM. Statistical analysis was performed with Stat View 4.5 software (Abacus Corporation, Baltimore, USA) for Windows, the data were analyzed using one-way ANOVA followed by Fisher’s protected least significance difference [PLSD] post hoc test. Significance level was accepted at $p < 0.05$.

Results:

The preliminary results came from testing the protective effect of glutathione against oxidative stress using RTCA system. Surprisingly, during all the preliminary tests, glutathione drastically decreased cell index. During these preliminary experiments glutathione was used at 10 mM, a rather high concentration. That unexpected result drove us to test lower concentrations in a dose-response manner. Figure 1 shows the effect of increasing concentrations of glutathione on the cell index in different cell types. Interestingly, all cell types, i.e. cancer cells (A549) as well as non cancer cells (3T3-L1, 13S24), showed a dose-dependent decrease of cell index in response to increasing concentrations of reduced glutathione, although the reduction of cell index was more or less marked according to cell line.

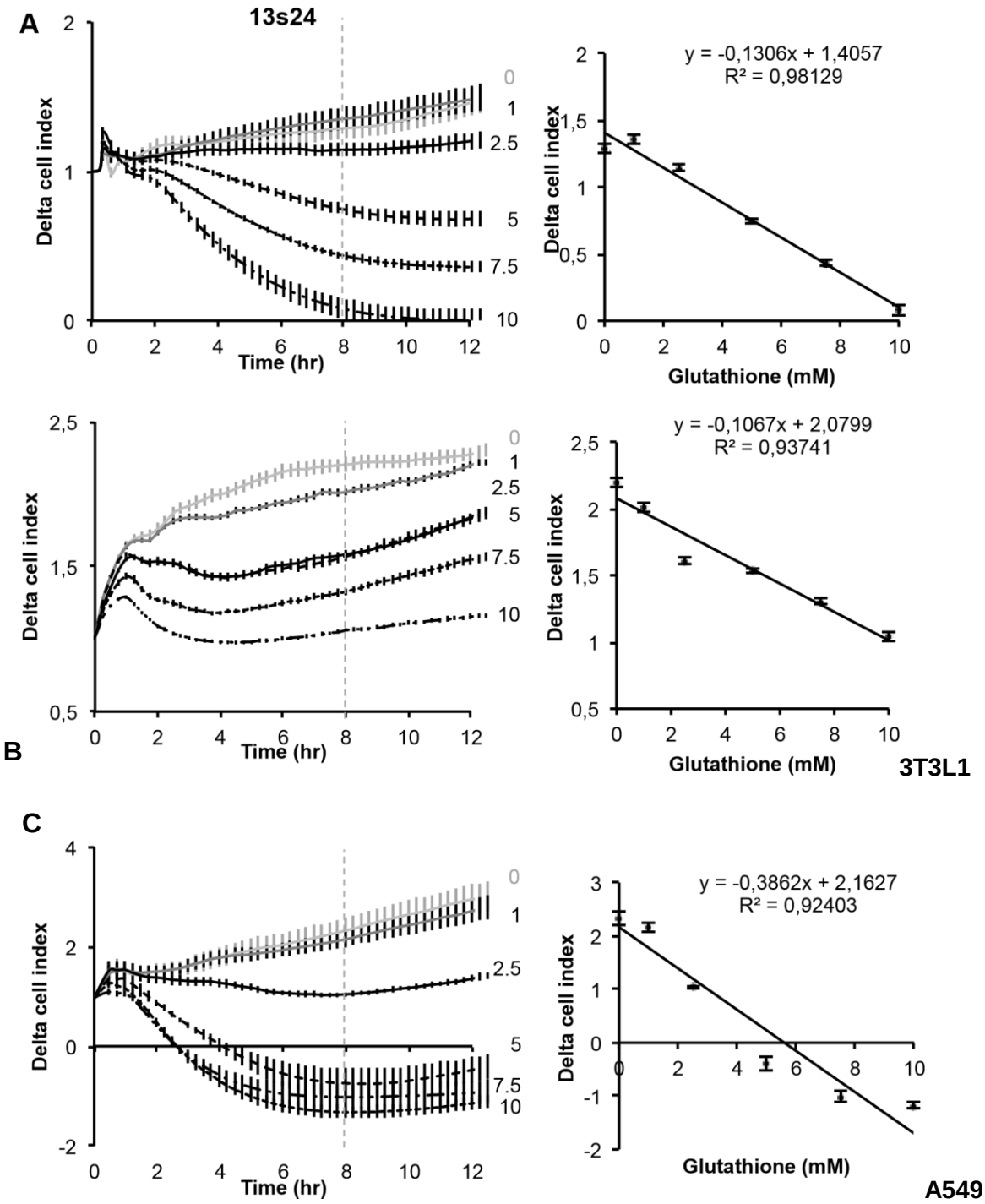

Figure 1: Real-time evolution of delta cell index in response to increasing concentrations of reduced glutathione on several cell lines A- 13s24 olfactory neuroblasts; B- 3T3-L1 C- A549 pulmonary cancer cells. Cell index was normalized at the time of treatment. Data are presented as mean delta cell indexes +/-SEM during time (left panel) or correlation curves 8 hours after treatment (right panels), n=8 replicates.

xCELLigence biosensor system allows the continuous measurement of cell index which is in fact an impedance, it depends on cell number and surface occupied by cells, and of adhesion

strength of cells to the substrate. Significant decrease in cell index may sign cell death or cell adhesion reduction. To further understand the effect of glutathione, we first checked whether glutathione decreased cell number. A 24 hour image acquisition showed that 10mM glutathione did not induced morphologic changes suggesting cell death. Figure 2 shows micrographs obtained before and 8, 16, 24 hours after exposure to 10 mM glutathione. No cell death is observed but instead a marked cell size reduction over time is obvious at 8, 16, 24 hours (Figures 2B, 2C, 2D). Futhermore, cell labeling with Zombie Red, a fluorescent marker of cells with permeable extracellular membrane cell related to cell death induction, confirmed that glutathione did not altered cell integrity even at high concentrations.

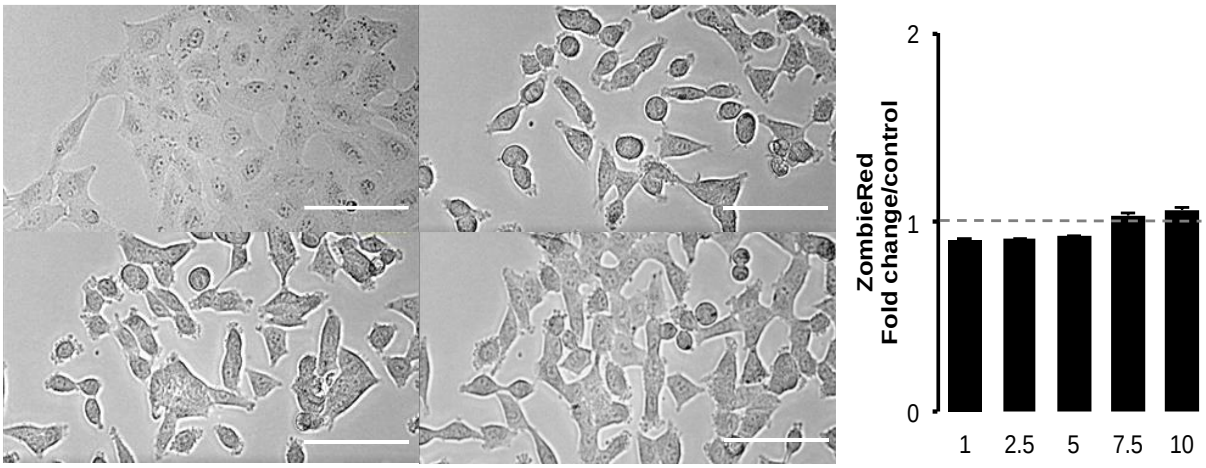

Figure 2: Morphological changes induced by glutathione in A549 cells are independent of toxicity. A: Contrast phase photomicrographs at times 0, 8h, 16h and 24h of a time-lapse in presence of 10 mM glutathione. No image of death cells is visible. Compare to time 0, cells reduced their size. Scale bar represents 100µM. B: Cell permeability to Zombie Red analyzed by fluorescence quantification in cytometry 2.5 hours after treatment, in a dose-dependent response to glutathione (mM). Data are presented as mean +/- fold changes Zombie Red fluorescence intensity in reduced GSH treated cells *versus* control media (n=4).

To further quantify cellular changes observed in response to glutathione we used the label-free single-cell analysis microscope Holomonitor (Phase Holographic Imaging, PHI, Lund, Sweden, Figure 3). The maximum optical thickness of cells significantly increased (Figure 3). Interestingly most of the increase occurs during the first 3 hours of glutathione exposure. As a consequence, cell volume increased markedly (Figure 3C).

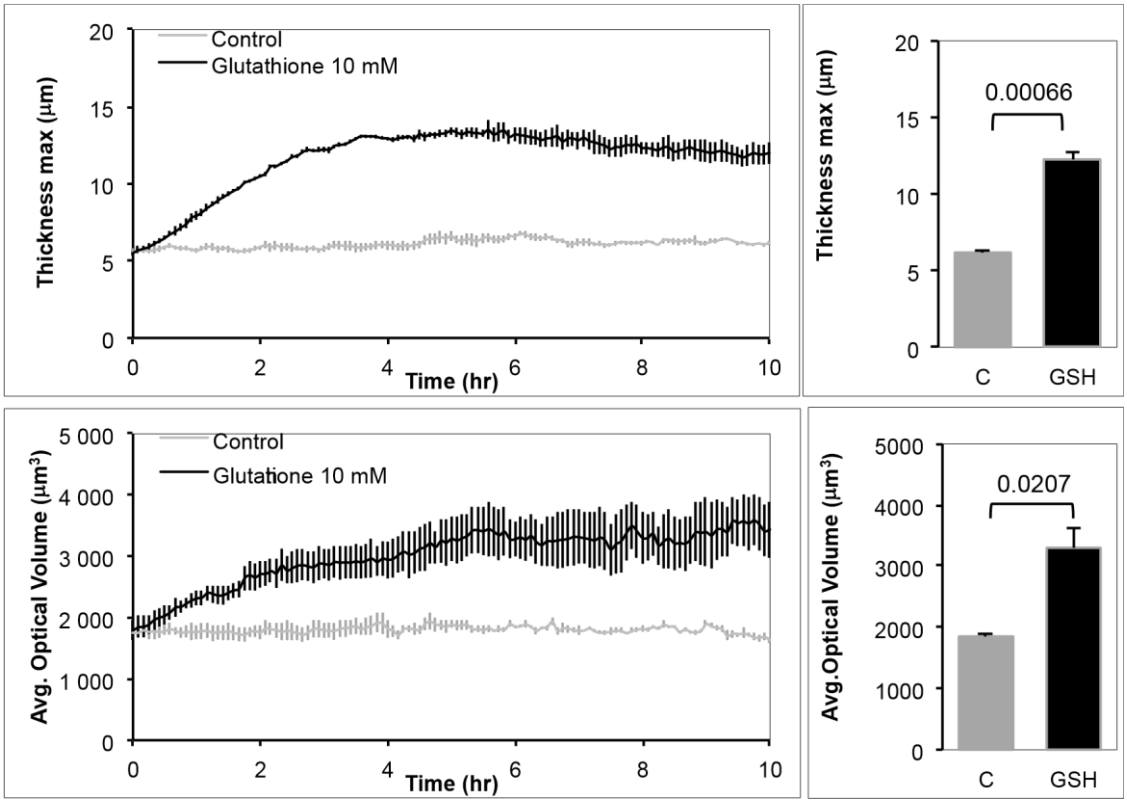

Figure 3: Real-time analysis of A549 cell size modifications induced by glutathione (10mM). Proliferative 459 cells were monitored on Holomonitor microscope during 10 hr (left panels). Maximum optical thickness (upper graph) and average optical volume (lower graph) were registered every 5 min. Data are presented as mean values +/-SEM (n=20 cells, 4 wells), at 8 h (left panel panels) with significant Student's t-test p-values ($p<0.05$) in treated (GSH) *versus* controls (C).

In the next experiment, buthionine sulfoximine (BSO, 10mM), the inhibitor of gammaglutamylcysteine synthetase was added during 24 hours to A549 cell culture (Figure 4). The inhibitor alone did modulate cell proliferation. It has no effect on glutathione action since the same inhibitory effect was observed in response to 10 mM in presence as well as in absence of BSO. Indeed, cell index reached values close to zero after glutathione addition independently of BSO. Within the next few hours, cell index increased slowly showing that cell were not dead. The fact that the inhibitor of gamma-glutamylcysteine synthetase did not alter the response of glutathione is of importance, since it demonstrates that the increase of intracellular concentration of glutathione is not required to reduce the cell index.

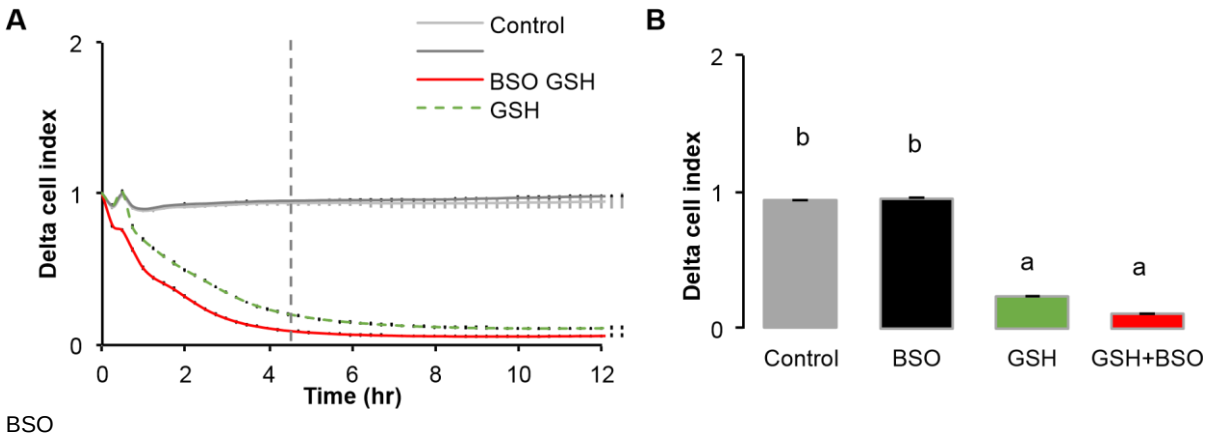

Figure 4: Realtime analysis of the effect of glutathione (10mM) in presence of gamma-glutamylcysteine synthetase (BSO at 10mM) on 459 cells. A549 cells were pre-incubated with BSO during 24 hr. Then glutathione 10mM was added either alone or in presence of BSO (10mM). A: the graph shows a similar response between cells pre-incubated in presence of BSO or not. B: histogram of delta cell indexes of control cells or cells exposed to BSO alone or GSH + BSO. Data are presented as mean delta cell indexes +/-SEM (n=8).

Since the previous experiment argued for an extracellular effect of glutathione, we next tested whether it may affect cell adhesion. Cell adhesion monitoring on xCELLigence has been previously demonstrated and can be assessed in short time experiments (Agaësse *et al.* 2016; El Kharbili *et al.* 2017). After trypsination, increasing concentrations of glutathione were added to cell suspension in DMEM. Then cell adhesion was measured on xCELLigence biosensor. Results show an inverse correlation between glutathione concentration and cell adhesion, the highest concentrations of glutathione corresponding to the lowest cell adhesion. Interestingly, the lower concentration of glutathione, 1mM increased cell adhesion compared to control (Figure 5A).

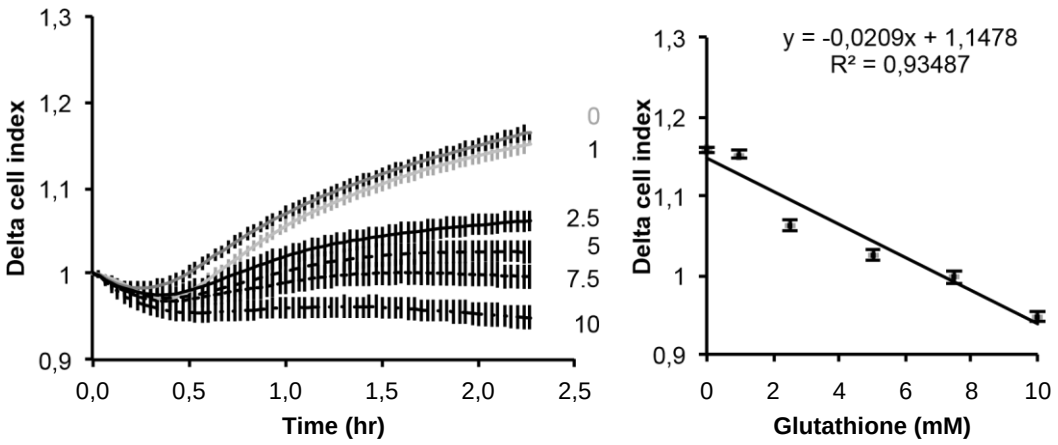

Figure 5: Real-time analysis of glutathione effect on A549 cell adhesion. A549 cells were loaded at 5,000 cells/cm^2. Results are presented as means of delta cell indexes ± SEM. Values have been normalized at the time of loading (left panel). Correlation curve between cell index and glutathione concentration is presented in right panel (n=8).

The decreased cell adhesion by glutathione may reflect intracellular modifications such as actin polymerization. To test that hypothesis we used phalloïdin, the toxin found in cap mushroom, known to bind specifically at the interface of F-actin subunits (Figure 5). FACS analysis of A549 cells labeled with fluorescent TRITC-phalloïdin shows a decreased incorporation of fluorescently labeled molecule following 24h glutathione treatment in A549 cells.

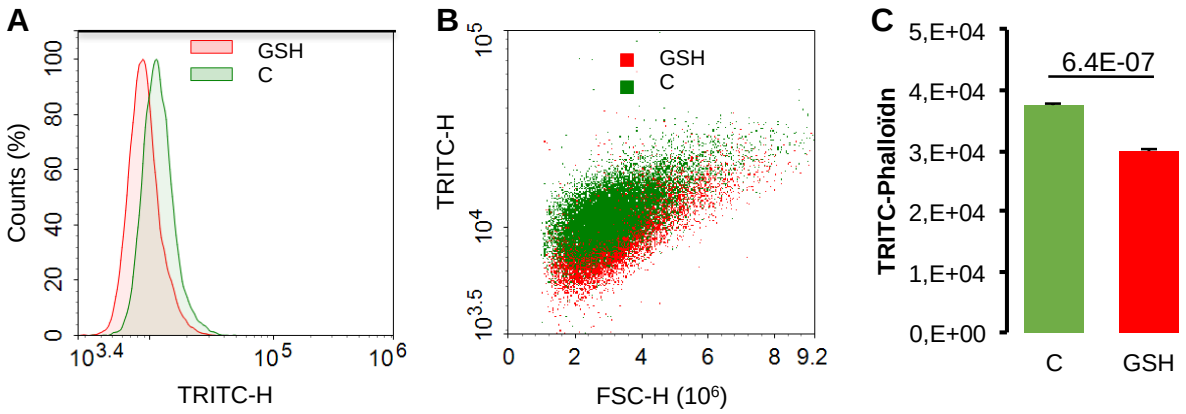

Figure 6: Quantification of TRITC-phalloïdin by cytometry in A549 cells exposed to glutathione (GSH 10 mM) during 24 hours. A- Representative histogram of cell number repartition according to fluorescence incorporation. B- Representative repartition of TRITC fluorescent cells according to cell size (FSC). C- TRITC-phalloïdin fluorescence quantification according to treatment (mean values +/- SEM, n=4) with significant Student t-test p-value ($p<0.05$).

DISCUSSION.

The present results show a marked effect of reduced glutathione on cell volume. Following the addition of reduced GSH in the culture medium, the decrease in cell index is fast and marked reaching almost basal values, occurring within 2-3 hours (Figure 1). That effect is observed on different cell types, with differences in the amplitude of cell index decrease, suggesting that the effect of glutathione is not cell specific but likely widely present in all cell types (and not related to cancer cells). The differences in cell responses suggest that cell equipment and/or sites of glutathione action are slightly different in quantity or in nature from one cell type to another. The cell index decrease is so strong that it suggests that cells are dying or even dead. Several observations attest that cells do not die. First is the visual control by microscopy and maintenance of cell membrane integrity (Figure 2). Second, the decrease in cell index is reversible. Indeed, after longer time exposure, cell index slowly increases despite the presence of glutathione and cell number was not affected. After washing out, the cell index increased markedly without fully reaching the control values (not shown). Third, real-time analysis using the quantitative phase contrast microscope Holomonitor M4, confirmed that there is no cell death (result no shown). Furthermore, we observed a marked increase in maximum optical thickness and in optical cell volume (Figure 3). On RTCA, the cell index depends on the surface occupied by cells but also on cell adhesion force. Since cell number is constant the only one explanation for the decreased cell index is that cell adhesion is affected by glutathione. A designed experiment on RTCA biosensor dedicated to measure cell adhesion shows a dose-response to glutathione on A549 cell adhesion, thus strongly arguing for a diminished cell adhesion force induced by glutathione, which was confirmed by a reduction of F-actin required for integrin clustering necessary for cell adhesion (review Kechagia *et al.* 2019).

Whether glutathione regulates cell adhesion through extracellular action rather than increased intracellular contents was supported by the extremely fast cell adhesion force reduction observed in real-time experiments. Indeed, a significant effect was detected within minutes even at low concentrations after glutathione addition (Figure 1). In order to test a possible effect of intracellular glutathione synthesis in such a process, buthionine sulfoximine (BSO), a specific the inhibitor of gamma-glutamylcysteine synthetase, was used to block the first step of intracellular glutathione synthesis. BSO at 10mM did not significantly affect the cell index of A549 cells. Pre-incubation of A549 during 24 hours with BSO before addition of glutathione, did not changed the decrease in cell index produced by reduced glutathione. It implies that

glutathione exerts its reducing effect on cell adhesion independently of intracellular pathways but rather through an activity at the extracellular level.

The present study did not solve the question of the mechanism involved in the action of reduced GSH on cells. Data from the literature show that N-acetylcysteine (NAC) is a disulphide breaking agent (Aldini et al. 2018). NAC produces similar effects as reduced glutathione on cell index and phalloïdin labeling (results not shown). Besides, glutathione is also reported to trigger the degradation of disulphide bonds (Koo et al. 2016). Disulfide bonds formed by the oxidation of two cysteine residues to give cystine are essential for the stability of secreted and plasma-membrane proteins (reviewed in Trivedi *et al*, 2009). Their cleavage is also of most importance to regulate secreted proteins such as thrombospondin, an extracellular glycocoprotein involved in cell-matrix adhesion. The role of disulfide bounds in the stability of focal adhesion proteins are largely depicted in the control of cell surface receptors (reviewed in Hogg *et al.* 2003) as well as the role of extracellular protein disulfide isomerases in the regulation of integrins, in platelet adhesion and thrombosis (reviewed in Mor-Cohen 2016) and more recently in cancer cell migration and invasiveness (Popielarski et al, 2019; Stojak et al, 2019). In breast cancer cells, the regulation of disulfide exchanges, mostly involving integrins, controls transendothelial migration of cancer cells. Such mechanism has been demonstrated using several free thiols blocking agents and/or protein disulfite isomerase inhibitors.

The effect of glutathione rises numerous questions. The concentrations of glutathione used in the present study are high. Some experiments have been performed with 10mM but several experiments have tested dose-responses effects from 1 to 10 mM. Significant effects of glutathione are observable at much lower concentrations than 10 mM, it is the case for RTCA cell index which is a highly sensitive method allowing to detect and quantify cell adhesion modifications in shorter times (few minutes) than fluorescence imaging nor cytometry in TRITC-phalloïdin labeled cells (2.5 hr). Several studies have reported rather high intracellular concentration of reduced glutathione, in liver cells it reaches 10 mM (Ballaroti *et al.* 2009, Fitzpatrick *et al.* 2012). Plasma and extracellular pools of reduced GSH are much lower than the intracellular pool, around 1-10 µM at the exception of bile (10 mM, Meister and Anderson 1983, Ballaroti et al. 2009) and alveolar lining fluid, 400-800 µM (Cantin et al. 1987, Stenzel et al. 1993). Present results also rise question on the true extracellular GSH concentration. Indeed, if reduced GSH combines with disulfide bridges on extracellular proteins, it then becomes obvious that its extracellular concentration drops, but then the extracellular GSH concentration takes into account only free GSH, which cannot be used as a reference for GSH

excreted by cells. The high intracellular concentration of reduced GSH and its fast binding on extracellular proteins suggests that cells can regulate their adhesion through excreted reduced glutathione. Transport of GSH out of cells was found in several cell types: human lymphoid cells, skin fibroblasts and macrophages (Meister and Anderson 1983). If cells like fibroblasts are able to export glutathione, it is most likely that most cell types are able to do it. As suggested by Meister and Anderson (1983), "Transport of GSH may protect the cell membrane against oxidative damage". Our results show that reduced glutathione is able to regulate cell adhesion and through adhesion, cell volume.

Export of GSH out of cells was observed in several cell types: human lymphoid cells, skin fibroblasts and macrophages (Meister and Anderson 1983) suggesting that it is a commune feature. It may reduce compounds in the immediate environment of the cell and might protect cells. It could also influence cell adhesion. Indeed, cell adhesion is mediated through integrins, a family of α/β heterodimeric molecules responsible for cell–matrix and cell–cell interactions. Integrins are subject to redox dependent conformational changes resulting in alterations of their binding activity (Eble et al. 2014). All integrins contain a large number of cysteine residues in their ectodomains. The breakage of disulfide bonds in integrins by reducing agents can induce their activation (Mor-Cohen et al., 2012; Yan and Smith, 2001).(Zhang). Integrin activation by thiol-disulfide was described on αVβ3, αIIbβ3, integrin subunit α11 [19] and β1 integrin [20]. Furthermore, the protein disulfide isomerase (PDI) directly interacts with integrins, mediating disulfide-thiol exchange, resulting in conformational changes and activation (Popierlarski et al. 2019). PDI can be found on the surface of several cell types, including fibroblasts (Popielarski et al. 2018). Adhesion of human skin fibroblasts to collagen and fibronectin is inhibited by para-chloro-mercuriphenyl sulfonic acid (pCMPS), a thiol blockers (Lahav 2000,.Rosenberg 2019).

In conclusion the present study demonstrates that reduced glutathione has a major impact on cell volume of several cell lines. That rises the hypothesis that reduced glutathione produced by cells can exert a direct control on their glycocalix which affect cell adhesion and cell volume. Such an hypothesis adds a new function of glutathione and most likely will necessitate a re-evaluation of cell adhesion functions according to their redox-state.

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