THE CHANGING FACES OF GLUTATHIONE, A CELLULAR PROTAGONIST

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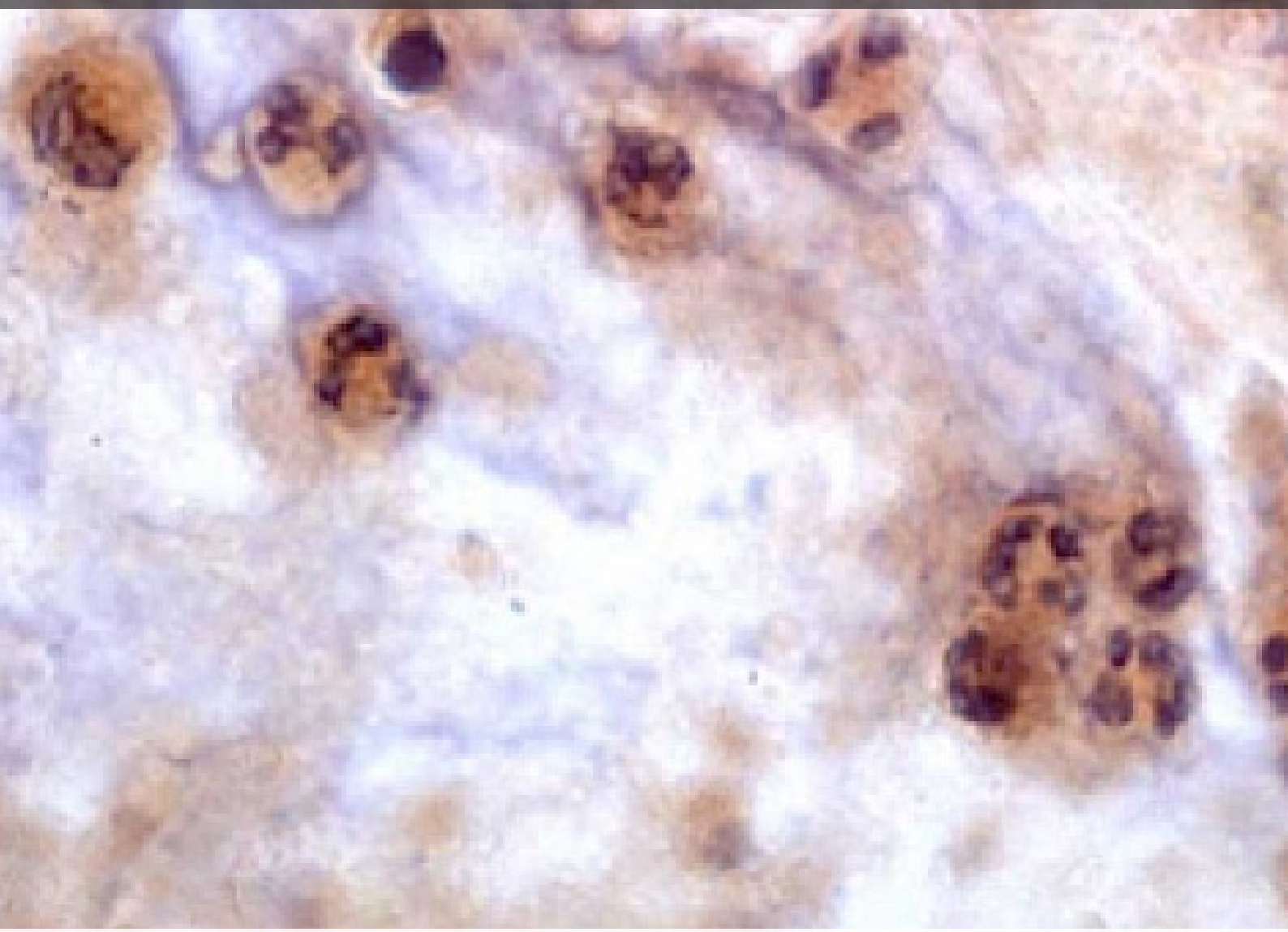

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THE CHANGING FACES OF GLUTATHIONE, A CELLULAR PROTAGONIST

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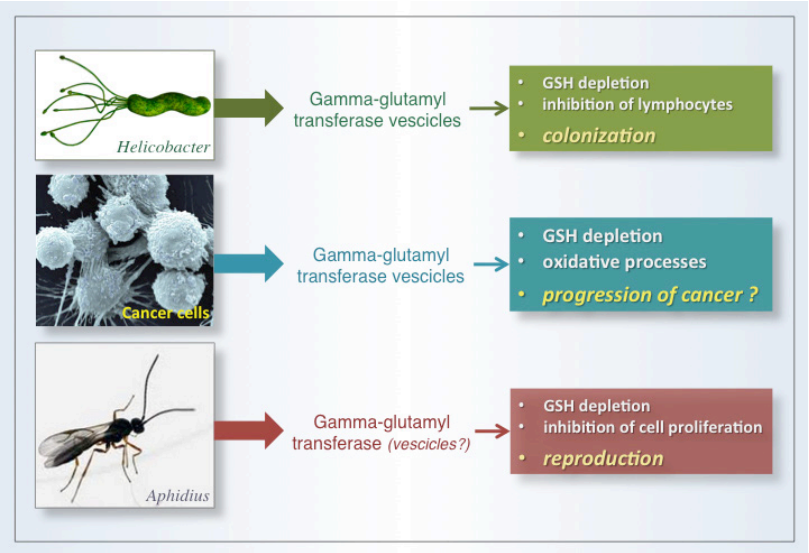
Glutathione (GSH) has been described for a long time just as a defensive reagent against the action of toxic xenobiotics (drugs, pollutants, carcinogens), both directly and as a cofactor for GSH transferases. As a prototype antioxidant, it has been involved in cell protection from the noxious effect of excess oxidant stress, both directly and as a cofactor of glutathione peroxidases. In addition, it has long been known that GSH is capable of forming disulfide bonds with cysteine residues of proteins, and the relevance of this mechanism ("S-glutathionylation") in regulation of protein function has been well documented in a number of research fields. Rather paradoxically, it has also been highlighted that GSH—and notably its catabolites, as originated by metabolism by gamma-glutamyltransferase—can promote oxidative processes, by participating in metal ion-mediated reactions eventually leading to formation of reactive oxygen species and free radicals. Also, a fundamental role of GSH has been recognized in the storage and transport of nitric oxide (NO), in the form of S-nitrosoglutathione (GSNO). The significance of GSH as a major factor in regulation of cell life, proliferation, and death, can be regarded as the integrated result of all these roles, as well as of more which are emerging in diverse fields of biology and pathophysiology. Against this background, modulation of GSH levels and GSH-related enzyme activities represents a fertile field for experimental pharmacology in numerous and diverse perspectives of animal, plant and microbiologic research. This research topic includes 14 articles, i.e. 4 Opinion Articles, 6 Reviews, and 4 Original Research Articles. The contributions by several distinguished research groups, each from his own standpoint of competence and expertise, provide a comprehensive and updated view over the diverse roles, the changing faces of GSH and GSH-related enzymes in cell's health, disease and death.



The invasion strategies of bacteria and other parasitic organisms can also target host glutathione (GSH) metabolism. Gamma-glutamyltransferase (GGT) activity of Helicobacter spp., the agents responsible of peptic ulcer, can trigger oxidative processes by mediating the metabolism and depletion of GSH in the host's cells. Bacteria release 'outer membrane vescicles'—submicroscopic structures resembling exosomes—which carry active GGT and can deliver the enzyme to cells of the gastric mucosa, thus producing GSH depletion and inhibition of mucosal immune system. Insect studies showed that the parasitic wasp Aphidius ervi injects into host aphids a venom containing large amounts of GGT, which triggers apoptosis in the upper part of the aphid ovarioles. Interestingly enough, GGT is expressed in a number of human malignancies and active enzyme can also be released from cancer cells in association with submicroscopic vesicles resembling exosomes. Such akin observations in evolutionarily distant organisms suggest that GGT/GSH-dependent processes may represent a convergent parasitic strategy, evolved with the same apparent biological aim: to perturb redox equilibria and impair immune defenses in host tissues, thus facilitating colonization and expansion of the invading cell populations. Image by Alfonso Pompella *(see Pennacchio et al., this volume, p. 114)*.

Cover image:
Gamma-glutamyltransferase activity (GGT, reddish stain) in granules of neutrophils crowding a sputum sample obtained from a cystic fibrosis patient during active lung inflammation. Abundant enzyme activity is also associated with mucus surrounding the cells. It has been reported that GGT can be released by activated neutrophils and other inflammatory cells, and can contribute to lung injury by depleting glutathione in bronchial excreates during lung inflammation (see this volume, article by Tuzova M, Jean JC, Hughey RP, Brown LAS, Cruikshank WW, Hiratake J and Joyce-Brady M (2014) Inhibiting lung lining fluid glutathione metabolism with GGsTop as a novel treatment for asthma. Front. Pharmacol. 5:179. doi: 10.3389/fphar.2014.00179). Cytochemical demonstration of GGT activity by gamma-glutamyl-4-methoxy-2-naphthylamide + Fast Garnet GBC reaction. Unpublished data by A. Corti, original magnification: 150x.

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EDITORIAL
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Editorial: the changing faces of glutathione, a cellular protagonist

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Keywords: glutathione, glutathione S-transferases, gamma-glutamyltransferase, oxidant stress, S-glutathionylation, redox regulation

Glutathione—the simple tripeptide composed of glutamic acid, cysteine, and glycine—is customarily defined as "*the most important intracellular non-enzymatic antioxidant,*" and most of the time such a swift definition is deemed sufficient to epitomize its functions in cellular homeostasis. But glutathione in fact is much more than that, as decades of passionate, unceasing research have documented. Just for instance, *extracellular* glutathione has also been shown to be of paramount importance in selected conditions—notably, respiratory pathophysiology—and functions of GSH other than antioxidant (indeed, even *prooxidant)* have been identified in years.

The title of the present e-Book explicitly recalls that of a fortunate commentary we wrote several years ago, back in 2003 (Pompella et al., 2003). During the decade or so since then, the interest in glutathione and related enzymes has continued to raise—slightly but steadily, as it had done in the decade preceding that publication (**Figure 1**). As also documented by the articles collected here, GSH biochemistry and pharmacology in fact involve wide and varied fields of interest in biology and medicine—from protein taxonomy to plant physiology, epigenetic regulation, cell energetics and the apoptosis/survival balance, carcinogenesis, drug resistance of cancer cells, inflammation-based diseases etc. It is thus very likely that experimental studies of diverse nature end up by encountering GSH at some earlier or later stage.

The present collection of papers can be a useful tool in order to feel the pulse of current glutathione research in several fields of biological and medical interest. To start with, the first two articles in the series both offer—from distinct points of view—intriguing conceptual re-evaluations of GSH biochemical significance. First of all, the Opinion paper by Prof. L. Flohé's group provides punctual considerations pointing out that the electrochemical potential of GSH cannot entirely describe its redox regulating functions, and other kinetics and thermodynamic factors may be needed for a more comprehensive appraisal. Enzyme catalysis should be regarded as prioritary vs. thermodynamic or electrochemical parameters in GSH-dependent redox events; in short—the way these authors put it—"*redox regulation by GSH needs enzymes*" (Berndt et al., 2014). On the other hand, the article which follows is an admirably straightforward and clear Original research paper. Basing on the results of simple *in vitro* experiments with hypochlorous acid (HOCl), Profs. Haenen and Bast conclude that the partially oxidized forms of GSH generated in the GSH-HOCl reaction also are effective antioxidants, and do substantially contribute to the overall scavenging activity. Therefore the latter is best measured by the *total number of reactive molecules scavenged,* rather than by the reaction rate of the GSH-HOCl reaction (Haenen and Bast, 2014).

The two subsequent articles deal with structural and evolutionary aspects. The first one, an Original research paper, is an intriguing exploration of the factors which may have contributed to the selection of redox-active disulfides during the evolution of protein structures. S-glutathionylation, and the action of glutaredoxin as well as thioredoxin, are among these factors. The authors select three proteins for detailed analysis—CD4, ERO1, and AKT—and with the help of phylogenetic trees propose how redox-active disulfides may appear into a protein structure during evolution, by stepwise mutation of two residues in the native sequence to cysteine (Mohanasundaram et al., 2015). The following Review article by Lallement et al. is also focused

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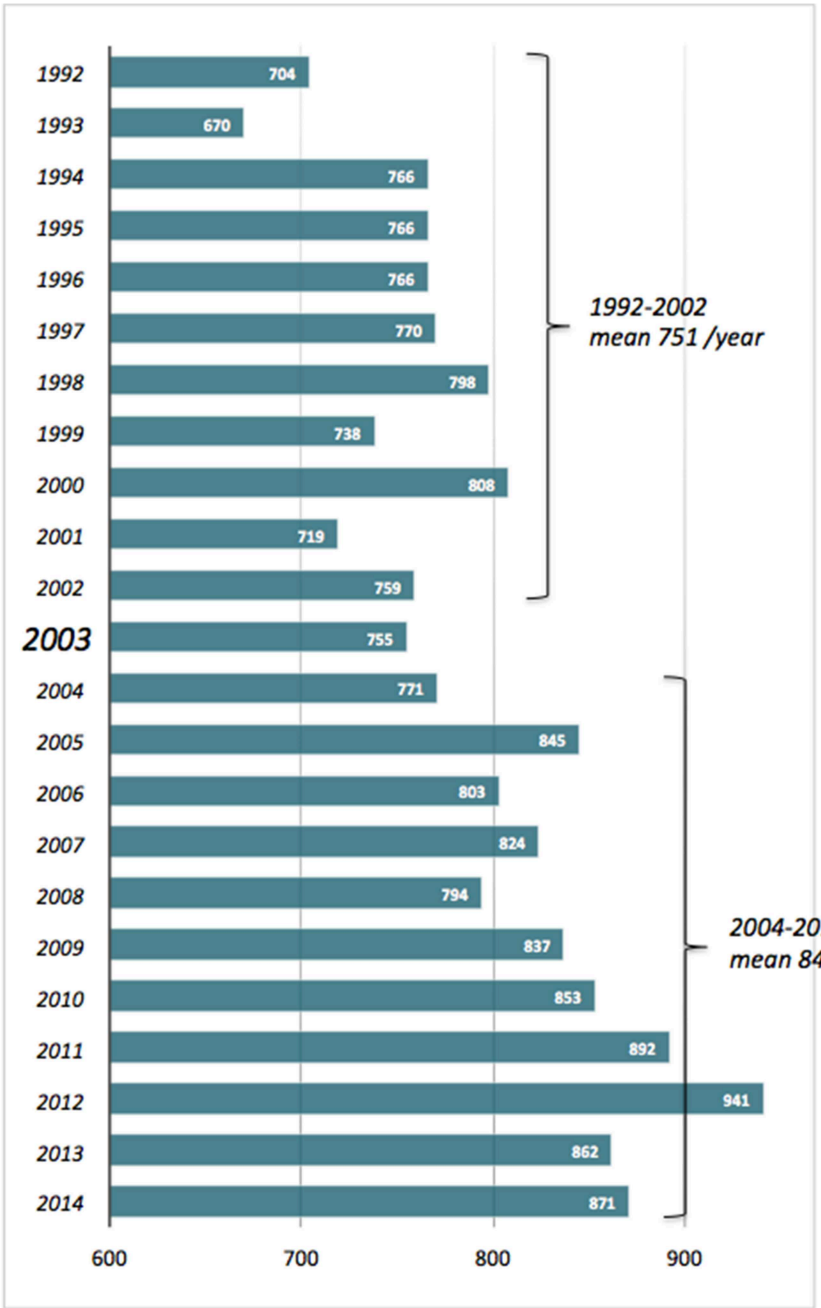

FIGURE 1 | No. of scientific papers published in the period 1992–2014 and quoting "glutathione" as title word. Source: Entrez PubMed, http://www.ncbi.nlm.nih.gov/pubmed?cmd=Search.

on evolutionary aspects, and in particular on cysteine-containing glutathione transferases (Cys-GSTs) of plants. These peculiar enzymes can effect protein de-glutathionylation, thus mimicking the action of glutaredoxin; but, apart from the obvious function of dehydroascorbate reductases in regeneration of ascorbic acid, the functions of other Cys-GSTs in plants remain obscure. The authors try here to shed some light, and indeed do a precious job by including a phylogenetic analysis of the 14 classes of plant GSTs and a comprehensive survey of all aspects involved: gene expression, structure, catalytic mechanisms, subcellular localization, potential physiological roles (Lallement et al., 2014).

Epigenetics is the subject of the following two papers. The Opinion article by Garcia-Giménez and Pallardò highlights the roles played by GSH in gene regulation, as it directly binds to cysteine residues in H3 histone and it regulates the activity of S-adenosyl methionine synthetase (MAT1A), a major enzyme involved in DNA methylation. The authors thus propose that GSH is a factor linking epigenetic regulation with the redox

status of the cell (García-Giménez and Pallardó, 2014). The subsequent Review is focused instead on the epigenetic regulation of glutathione S-transferase P1 (GSTP1), an ezyme activity also provided with "caretaker" function. Inactivation of this gene is often observed in human neoplasia (prostate, breast, and liver cancer, as well as leukemias), and is the result of acquired somatic CpG island promoter hypermethylation. The authors underscore the possibility of using such GSTP1 epigenetic alterations as biomarkers for early diagnosis (of prostate cancer in the first place), as well as potential targets of preventive or therapeutic treatments (Schnekenburger et al., 2014).

GSH of mitochondria plays a defensive role against reactive oxygen species deriving from respiration, and regulation of its levels impacts on the permeabilization of mitochondrial membrane, i.e., the first step in several instances of programmed cell death. This is the subject of the subsequent two Reviews and one Opinion article. The first paper (Ribas et al., 2014) includes a comprehensive appraisal of the involvement of mitochondrial GSH alterations in several important human pathologies, as well as a survey of the available strategies capable of restoring the mitochondrial pool of GSH and/or other antioxidants. The second Review evaluates the involvement of S-glutathionylation and/or thiols redox modulation in cell signaling related to nitric oxide induced responses, autophagic pathways and viral infection (Aquilano et al., 2014), while the Opinion paper which follows is an interesting reappraisal of studies showing how apoptosis can be linked to an intracellular oxidant stress consequent to active GSH extrusion, leading to formation of inter-protein disulfides implicated in the proapoptotic signaling. The authors note that GSH depletion may promote either pro-apoptotic or pro-survival pathways, depending on its kinetics of depletion, and speculate that the reactive cysteines of proteins involved in the opposite responses (e.g., cell-protective NF-kB component p50 vs. pro-apoptotic Bax) may be differently modulated according to the swiftness of the redox imbalance (De Nicola and Ghibelli, 2014).

The two subsequent Original research papers are dedicated to inflammation and immune-related diseases. The first one reports data confirming that erythrocytes of HIV patients show decreased GSH levels, an effect known to be due to HIV-1 transactivator protein (TAT) altering the expression of GSH biosynthetic enzymes (Choi et al., 2000). The authors conclude that resupplementing intracellular GSH by means of liposomal formulations may be effective in restoring immune functions, such as e.g., the antimycobacterial activity in macrophages from HIV patients (Morris et al., 2014). The other Original research paper in this subsection is concerned with an aspect of high relevance in respiratory medicine, i.e., the fundamental antioxidant and antiinflammatory role played by GSH contained in the epithelial lining fluid of airways. These levels are subject to the catabolizing activity of gamma-glutamyltransferase (GGT), an enzyme increasing in airways during inflammation. The authors elegantly demonstrate—in the IL-13 model of allergic airways inflammation in the mouse—that the pharmacological inhibition of airways GGT by means of a synthetic substrate analog, GGsTop (Han et al., 2007) results in a remarkable sparing of *extracellular GSH*, and can therefore augment antioxidant defenses and protect lung parenchyma against the prooxidant insults deriving from accumulation of activated inflammatory cells (Tuzova et al., 2014). The described approach might prove indeed valuable in lung diseases that perturb the extracellular lining fluid GSH pool, such as cystic fibrosis, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease.

The conclusive three papers in our e-Book deal with cancer-related issues. The Review article by Prof. S. Toyokuni is a survey of the factors affecting the complex interplay of cellular transport systems for iron with those in charge of cellular supply of cysteines (Toyokuni, 2014). Excess iron is strictly linked with promotion of carcinogenesis, and on the other hand, the expression of the cystine/glutamate antiporter is intimately associated with ferroptosis, an iron-dependent, non-apoptotic form of cell death observed in cancer cells. The role of Nrf2/Keap1 signaling pathway—one of the most important cell defense and survival pathways—is discussed, as well as the recent finding that cancer stem cells in certain neoplasias present a stabilization of the xCT subunit of the x_c^- cystine/glutamate antiporter, leading to increased intracellular GSH levels. This may explain the robustness of cancer cell defenses against oxidative stress, and is likely implicated in resistance to chemotherapeutic agents (Ishimoto et al., 2011).

The paper which follows also is a Review article, and focuses on the expression of glutathione S-transferases (GSTs) as well as of GGT as factors in the promotion of a more aggressive and resistant phenotype of cancer cells. GGT activity allows cells to maintain/reconstitute their GSH supplies which are utilized by GSTs for conjugation of xenobiotics, and in this way both enzyme activities synergistically contribute to detoxification of anticancer drugs. Nevertheless, pro-drugs have been synthesized that can be selectively *activated* by GSTs and GGT, whose expression is thus turned into a factor of *vulnerability* of the cancer cell. The Review recapitulates the different drug strategies involving GSH-conjugates and enzymes of the mercapturic acid pathway employed over the last two decades (Ramsay and Dilda, 2014). Amongst the long list of compounds investigated, a limited number of GSH-conjugates have recently progressed toward clinical trials, including both GST-activated nitrogen mustard TLK286 and GGT-activated arsenic-based prodrugs GSAO and Darinaparsin.

The very last paper of the e-Book also is the most speculative contribution to this series. In their Opinion paper Pennacchio et al. (2014) start from the observation that selected bacterial strains *(Helicobacter* spp. in the first place, plus others) can secrete active GGT enzyme, which causes depletion of GSH in cells of infected epithelia. The resulting oxidant stress has been put in connection with the invading ability of bacteria, i.e., with their virulence (Gong et al., 2010). A very similar phenomenon has been observed with selected parasitic insects, capable to secrete a GGT-containing venom capable of inducing GSH depletion in tissues of parasitized organisms. The authors highlight that several human neoplastic cell lines have also been shown to secrete active GGT enzyme, in the form of exosome-like vescicles (Franzini et al., 2009) resembling those secreted by *Helicobacter* (Zhang et al., 2013). It is thus proposed that the phenomenon may represent a true evolutionarily convergent

strategy, spanning across very diverse life forms, aiming at depleting GSH levels in order to impair host's redox regulation and defenses to facilitate invasion and colonization. In this perspective cancer cells could be regarded as just another species of invasive "microrganisms," and such a view appears consistent with other recent, unrelated observations pointing at the fact that cancer might conceivably be treated like an *infectious disease,* by even repurposing approved antibiotics for anticarcer therapy, across multiple tumor types (Lamb et al., 2015).

In summary, the articles collected in the present e-Book represent an assortment of updated data, hypotheses and interpretations deriving from a pretty wide spectrum of precincts in current biomedical research. This was somewhat expected, due to the exquisite interdisciplinarity of the subject, and in some way even desired, to fully exploit the informative potential typically owned by an online publication. Rather, as a matter of fact, it is felt that soon enough there might again be the need of a Topic and e-Book on glutathione—this seemingly inexhaustible *cellular protagonist.*

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OPINION ARTICLE
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Redox regulation by glutathione needs enzymes

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The GSH/GSSG redox potential has become a fashionable electrochemical parameter believed to be a major driving force of redox reactions regulating biological events (Schafer and Buettner, 2001; Jones, 2006; Blanco et al., 2007; Chaiswing et al., 2012). Here, we will challenge this concept, because we consider it an untenable simplification that ignores kinetic constrains and detracts the attention from more important, though more complex, catalytic events. The focus of this article is the importance of reaction kinetics vs. thermodynamics in the redox regulation of biological systems.

THE IMPACT OF GLUTATHIONE ON BIOLOGICAL REDOX EVENTS

Whoever tried to directly determine redox potentials of proteins electrochemically will not forget the boring minutes or hours of waiting until the needle of the potentiometer had come to rest. In order to obtain any reliable read-out in reasonable time, a low molecular redox mediator is almost regularly required to enable an electron transfer between the macromolecule and the electrode and, of course, access of oxygen has to be strictly prevented. The physiological relevance of an electrochemical parameter measured under such artificial conditions may be questioned. If the redox potential of a thiol/disulfide couple is to be determined, problems already show up with low molecular mass compounds such as GSH or cysteine, since they inactivate all metal electrodes (Jocelyn, 1967). In fact, standard potentials E_0 or midpoint potentials at defined conditions (e.g., E_{m7} at pH 7) of such compounds are usually not determined directly, but estimated by means of the Nernst equation from concentration changes after equilibration with other redox couples of seemingly known standard potential (Rall and Lehninger, 1952; Eldjarn and Pihl, 1957; Rost and Rapoport, 1964; Van Laer et al., 2013). Rost and Rapoport cynically compiled the GSH/GSSG potentials measured up to 1964: The E_{m7} values ranged from −350 to +40 mV depending on the methodology applied (Rost and Rapoport, 1964). With their own value of −240 mV, which was based on the spontaneous equilibration with the NADH/NAD redox couple, they nicely comply with the E_{m7} which is at present dogmatically accepted, although method sensitivity remains a problem (Van Laer et al., 2013). Calculation of the actual potential in biological samples from concentration measurements is further complicated by vague estimations of subcellular compartment volumes and artifacts occurring during sample work-up. In contrast, indicator systems that specifically sense particular redox couples allow real-time observation of redox changes (Gutscher et al., 2008) and have more recently disclosed cases of unexpected subcellular distribution (Kojer et al., 2012; Morgan et al., 2013). In respect to quantitative results, however, this promising approach has its inherent limitations.

The experimental difficulties to obtain reliable potentials of thiol/disulfide systems prompt further concern to accept these parameters or changes thereof as critical determinants of biological events. For sure, standard redox potentials, with appropriate consideration of pH, temperature, and concentration effects, can tell us in which direction a reaction between different redox couples might go. However, it does not disclose how fast the reaction will be or whether it will ever happen within a biologically relevant time span. Unlike fast equilibration of inorganic redox systems such as couples of transition metals, oxidation–reduction reactions of organic molecules usually face a barrier of activation energy, which can be even prohibitory. Therefore, redox potentials do not translate into reaction velocities and nature does typically not rely on spontaneous equilibration between redox couples but on enzymatic catalysis. Revealingly, one of the first attempts to get an idea on the midpoint potential of the GSH/GSSG couple back in 1952 made use of enzymatic catalysis (Rall and Lehninger, 1952): The NADPH/NADP did simply not react with the GSH system until a then newly discovered enzyme, glutathione reductase, was added to the reaction mixture. Equally revealing was the observation that the NADH/NAD couple, which slowly interacts with the GSH system (Rost and Rapoport, 1964), could not substitute for NADPH/NADP in the enzyme-catalyzed system (Rall and Lehninger, 1952), although the redox potentials of the two nucleotide couples are practically identical. The enzyme, thus, contributed two pivotal aspects that characterize reactions in living organisms: adequate reaction velocity and appropriate specificity. In chemical terms, life is as a metastable system composed of many potential reaction partners. These,

however, do not promiscuously react with each other according to their Gibbs free energy ΔG or Nernst potential ΔE. Instead, activation energy barriers largely prevent their interaction and, thus, the approach to equilibrium (Flohé, 2013). For the same reasons outlined above, calling glutathione a redox buffer is misleading. Unlike an inorganic pH buffer, which binds and releases protons without any catalytic support, the GSH/GSSG couple does not pick up or releases redox equivalents spontaneously at relevant velocity. Like an inorganic pH buffer, the capacity of the couple to (indirectly) buffer cellular redox changes depends on the concentration of GSH and GSSG, respectively. However, these concentrations are anything else but static, but steady-states that, again, are kinetically controlled by enzymes utilizing or regenerating GSH. Therefore, it is the privilege of enzymes to determine the capacity of GSH-mediated redox buffering and to lower the activation energy in a specific and regulated way to sustain vital functions and simultaneously conserve the overall high energy level of the metastable condition called life.

ENZYME-BASED REDOX SIGNALING

Signaling requires the reversible modification of a sensor and the subsequent activation of transducer and effector molecules (**Figure 1**). These events are reversed by modulators that turn off or degrade these signaling molecules and a negative feedback inhibition that modulates the signal itself. In order to function in spatio-temporally controlled signaling events, most of these reactions need to be catalyzed by enzymes to reach the required reaction velocities and specificities. Redox signaling is based on reversible oxidative posttranslational modifications such as thiol-disulfide switches, S-glutathionylation, and S-nitrosylation.

S-glutathionylation of many regulatory proteins (Pompella et al., 2003; Yin et al., 2012; Demasi et al., 2013; Ghezzi, 2013), might indicate a direct impact of the GSH/GSSG couple on redox regulation. It was therefore tempting to speculate that changes in the cellular GSH/GSSG ratio or its electrochemical correlate, the pertinent redox potential E, directly affects the redox state and function of redox-sensitive regulatory proteins. This view, however, implies that the glutathione system easily equilibrates with protein thiols, which is not the case. Posttranslational redox modifications occur only at specific cysteinyl residues, in response to specific stimuli and not randomly. As outlined above, thermodynmics, i.e., ΔG or redox potentials, do not determine reaction velocities. *In vivo*, these are controlled through the regulation of enzyme activity. By analogy, protein (de)-phosphorylation, albeit thermodynamically favorable, is not controlled by the ΔG for ATP hydrolysis or Atkinson's highly quoted "energy charge" (Atkinson and Fall, 1967; Atkinson and Walton, 1967), but needs to be catalyzed by kinases and phosphatases to reach the required reaction velocities and specificity. Why should specific redox modifications of proteins not equally require catalysis? The spontaneous equilibration of protein thiols with the GSH/GSSG couple would be both too slow and too unspecific and, thus, not practical in signaling events. Not surprisingly, S-glutathionylation, which appears not only under conditions of oxidative or nitrosative stress, but also under physiological conditions without dramatic changes in the GSH/GSSG ratio appears to be dependent on enzymatic activities. Enzymes of the thioredoxin family, especially glutaredoxins (Grxs), efficiently catalyze de-glutathionylation and both glutaredoxins and glutathione S-transferases have been shown to promote S-glutathionylation (Gravina and Mieyal, 1993; Lillig et al., 2008; Townsend et al., 2009; Menon and Board, 2013). Organisms with low or no glutathione but analogous posttranscriptional modifications, i.e., S-mycothiolation or S-bacillithiolation, evolved specific enzymes such as mycoredoxins and bacilliredoxins (Van Laer et al., 2012; Gaballa et al., 2014).

EXAMPLES OF REDOX-REGULATED PATHWAYS

Many cellular functions have been already associated with redox regulation. Although just a small fraction of the 214,000 cysteines encoded in the human genome (Go and Jones, 2013) fulfill the prerequisites for thiol redox signaling, Dean Jones calculated that every cellular pathway harbors at least one redox sensitive element. In line with the above reasoning, not a single cellular pathway has been documented to be dependent on the GSH/GSSG ratio without involvement of any enzymatic activity. Specific thiol redox signaling based on GSH-utilizing enzymes has been identified in the context of numerous biological functions. Glutaredoxins are involved in DNA synthesis via regulation of ribonucleotide reductase (Sengupta and Holmgren, 2014), assimilatory sulfate reduction via regulation of phosphoadenylylsulfate reductase (Lillig et al., 2003), apoptosis via regulation of signaling molecules such as Fas or procaspase-3 (Allen and Mieyal, 2012), vessel formation via regulation of sirtuin 1 (Bräutigam et al., 2013), and many others in all kingdoms of life. Glutathione peroxidases regulate insulin signaling (GPx1) (McClung et al., 2004), NF-κB activation (GPx1 and 4) (Kretz-Remy et al., 1996; Brigelius-Flohé et al., 2000), lipoxygenase-triggered apoptosis (GPx4) (Brigelius-Flohé et al., 2000; Seiler et al., 2008), and adaptive responses (yeast GPx) (Delaunay et al., 2002). Here, we present in more detail two examples of enzyme-operated protein thiol switches (**Figure 1**).

The first described example of redox-regulated signaling is the regulation of the OxyR transcription factor in procaryotes. The signaling molecule H_2O_2 oxidizes cysteine 199 (Aslund et al., 1999) turning OxyR into a transducer and subsequently via binding of the corresponding responsive DNA element into an effector. Only oxidized OxyR activates expression of genes encoding proteins involved in defense against oxidative stress (Storz et al., 1990). Increased levels of alkyl hydroperoxide reductase AhpC/AhpF inactivate OxyR induced transcription by removing the signal molecule H_2O_2. Activity of OxyR can be modulated, i.e., terminated, by Grx-catalyzed reduction. GSH is required for the regeneration of reduced Grx.

In vertebrates, axonal guidance during embryonic development and regeneration depends on extracellular signaling molecules. Semaphorin 3A is such a repulsive signal, detected by the plexin1/neuropilin receptor pair. Subsequently, the signal is transferred to collapsin response mediator protein 2 (CRMP2) that regulates cytoskeletal

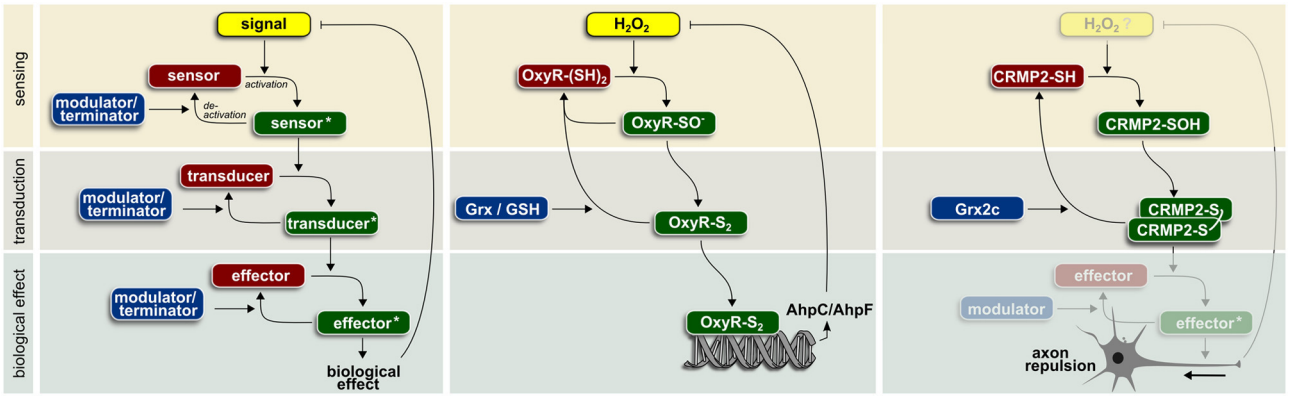

FIGURE 1 | Enzyme-based redox signaling in biological systems (for details see text).

organization and thereby axonal outgrowth/repulsion. The biological activity of CRMP2 depends on posttranslational modifications. Redox regulation of CRMP2 during development of the zebrafish brain requires activity of the vertebrate-specific Grx2 (Bräutigam et al., 2011). Knock-down of Grx2 inhibited the formation of an axonal scaffold and led to the loss of virtually all types of neurons in zebrafish. Remarkably, a change in the overall redox potential based on Grx2 knock-down was not observed. Overexpression of the corresponding isoform, cytosolic Grx2c, in a human cellular model of neuronal differentiation increased both the length and number of branching points of neurites (Bräutigam et al., 2011). *In vitro* analyses demonstrated a Cys504-Cys504 thiol-disulfide switch that determines distinct conformations of the homotetrameric protein (Gellert et al., 2013). This disulfide/thiol switch is operated by cytosolic Grx2 as modulator/terminator (Bräutigam et al., 2011; Gellert et al., 2013). Notably, incubation with excess GSSG alone could not trigger this switch (Gellert et al., 2013). Instead, oxidation of CRMP2 could be the result of the specific, semaphorin 3A-induced H_2O_2 generation through the monooxygenase MICAL (Morinaka et al., 2011).

CONCLUSION

The intention of this article was to underscore the priority of enzyme catalysis vs. thermodynamic or electrochemical parameters in GSH-dependent redox events. Although any kind of kinetically competitive reaction may interfere with a slow equilibration between redox couples, enzymatic ones are the most likely candidates. For example, thiols, in particular GSH, easily reduce H_2O_2. However, the bimolecular rate constants for the spontaneous reactions of low molecular mass thiols with hydroperoxides hardly reach $30\,M^{-1}s^{-1}$ (Winterbourn and Metodiewa, 1999; Van Laer et al., 2013), whereas those of the peroxidatic cysteines or selenocysteines in enzymes reach 10^7 and 10^8 $M^{-1}\,s^{-1}$, respectively (Trujillo et al., 2007; Toppo et al., 2009). Collectively, the above mentioned examples indicate that the GSH/GSSG redox potential is not likely the magic force that by itself steers biological events. Rather are potential changes, as observed under pathological conditions, the consequence of metabolic disturbances such as deficiencies or exhausted capacity of enzymes that require GSH or other thiols as substrates. If this assumption turns out to be correct, GSH-related biological reactions should not follow the concentration dependence predicted by the Nernst equation, but comply with the kinetic characteristics of the enzymes involved (Flohé, 2013).

AUTHOR CONTRIBUTIONS

All authors jointly wrote the manuscript.

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Glutathione revisited: a better scavenger than previously thought

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Glutathione (GSH) is the classical example of a scavenging antioxidant. It forms the first line of defense and efficiently scavenges reactive species, e.g., hypochlorous acid (HOCl), before they inflict damage to biomolecules. Scavenging antioxidant activity is best established in competition assays (that closely mimics molecular mechanism of the biological effect). In this type of assay, the antioxidant competes with a molecule that functions as an easy read-out detector for a reactive species. It is generally assumed that the scavenging antioxidant activity reflects the reaction rate constant of the antioxidant with the reactive species (k_a). However, critical appraisal of several competition assays of GSH with HOCl as reactive species, reveals that k_a does not determine the scavenging antioxidant activity. Assays using acetylcholine esterase, alpha1-antiprotease, methionine, and albumin as detector are compared. The total number of molecules of the reactive species scavenged by GSH plus that by partially oxidized forms of the GSH, reflect the scavenging activity of GSH. The contribution of the partially oxidized forms of GSH depends on the reactivity of the competing molecule. In several assays the partially oxidized forms of GSH have a substantial contribution to the scavenging activity of GSH. In contrast to the prevailing perception, not the reaction rate but rather the total number of molecules of the reactive species scavenged reflects the true scavenging activity of an antioxidant like GSH.

Keywords: glutathione, antioxidant, hypochlorous acid, alpha 1-antitrypsin, acetylcholinesterase, rate constant

INTRODUCTION

Many diseases are associated with the production of reactive oxidizing species that damage physiologically essential molecules. The classical view is that antioxidants scavenge these reactive oxidizing molecules and thus offer protection against disease. Antioxidants can be either enzymatic or non-enzymatic in nature. Together they form an elaborate web and protect the organism from widespread oxidative damage. Moreover, non-enzymatic antioxidants which are frequently identified in the diet are major constituents of food supplements or are administered as drugs. Antioxidant activity of compounds is commonly determined by means of competition assays. Based on these *in vitro* assays effective antioxidants are selected for further development and for further human use. Recently, doubts have been raised as to the *in vivo* relevance of antioxidants in disease protection in general. Several misconceptions on antioxidants have been uncovered (Bast and Haenen, 2013).

What thus far undisputedly remained is the competition assay as an unquestionable way to identify and categorize either endogenous or exogenous antioxidants. Similarly, the antioxidant action of cellular glutathione (GSH) in competitive protection against redox damage is also undeniable. GSH competes with other cellular redox active biomolecules for oxidizing reactive species and thus is a vital cellular protagonist.

In competition assays, the antioxidant (A) competes with a detector molecule (D) for the reactive oxidizing species (R). In other words, the antioxidant (A) protects the detector molecule (D) by scavenging the reactive species (R). The detector molecule (D) of course mimics the biomolecule which can be affected by R

$$\begin{aligned} &+\mathrm{A} \xrightarrow{k_a} \text{oxidized antioxidant} \\ \mathrm{R} \\ &+\mathrm{D} \xrightarrow{k_d} \text{oxidized detector.} \end{aligned}$$

From this scheme it can be derived that the rate of radical scavenging by A is proportional to the rate constant k_a of the reaction of A with R and the concentration of A.

Comparison and critical evaluation of various competition assays revealed that not the reaction rate constant of GSH with the reactive species determines its scavenging antioxidant activity, but rather the total number of molecules of the reactive species scavenged, reflect the true scavenging activity of the antioxidant GSH.

MATERIALS AND METHODS

MATERIALS

Reduced GSH, sodium hypochlorite, L-methionine, and 1,4-dithiotreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. All experiments were performed in 145 mM potassium phosphate buffer pH 7.4.

METHODS

Determination of stoichiometry and capacity

The stoichiometry of the antioxidant (here either GSH or 1,4-dithiothreitol) is defined as the number of molecules of reactive

species that react directly with one molecule of antioxidant. The observed stoichiometry was quantified by determining the concentration of the antioxidant immediately (within 30 s) after addition of a relatively small amount of HOCl. The remaining concentration of the antioxidant was plotted against the initial concentration of HOCl and the data were fitted linearly. The intercept of the linear fit with the x-axis was divided by the initial concentration of antioxidant to obtain the observed stoichiometry. It should be noted that the observed stoichiometry, determined by this procedure, includes the products that react much faster with the reactive species than does the parent compound and might deviate from the actual stoichiometry.

The total scavenging capacity is defined as the number of molecules of HOCl that are scavenged by the antioxidant within a certain time span, when an excess of HOCl is added to the antioxidant. The total scavenging capacity is the sum of the stoichiometry of the parent antioxidant, of the first product and of all other products formed in subsequent reactions. The total scavenging capacity of the antioxidants (GSH or 1,4-dithiothreitol) was determined by adding HOCl in a concentration range of 0–10 mM to a fixed concentration of the antioxidant of 1 mM. Thirty seconds after adding HOCl, the concentration of remaining HOCl that was in excess, was determined spectrophotometrically. The remaining concentration of HOCl was plotted against the initial concentration of reactive species and the data was fitted linearly. The intercept with the x-axis was divided by the initial concentration of the antioxidant to obtain the total scavenging capacity. The procedure to determine the observed stoichiometry and total scavenging capacity of 1,4-dithiothreitol is illustrated in the inset of **Figure 1**.

The chemical nature of the conducted experiments results in very small errors, which have not been shown for sake of clarity.

Competition assays

In the competition assay with methionine as detector in a final concentration of 1 mM of methionine was used. Different concentrations of the GSH were added to the methionine containing solution. The mixture of the detector (methionine) and the antioxidant (GSH) was pre-incubated at 37°C for 5 min. During rigorous mixing, HOCl was added (final concentration of 0.63 mM). After completion of the reaction, the remaining amount of the antioxidant GSH was determined (Peskin and Winterbourn, 2001). The EC_{50} is defined as the concentration

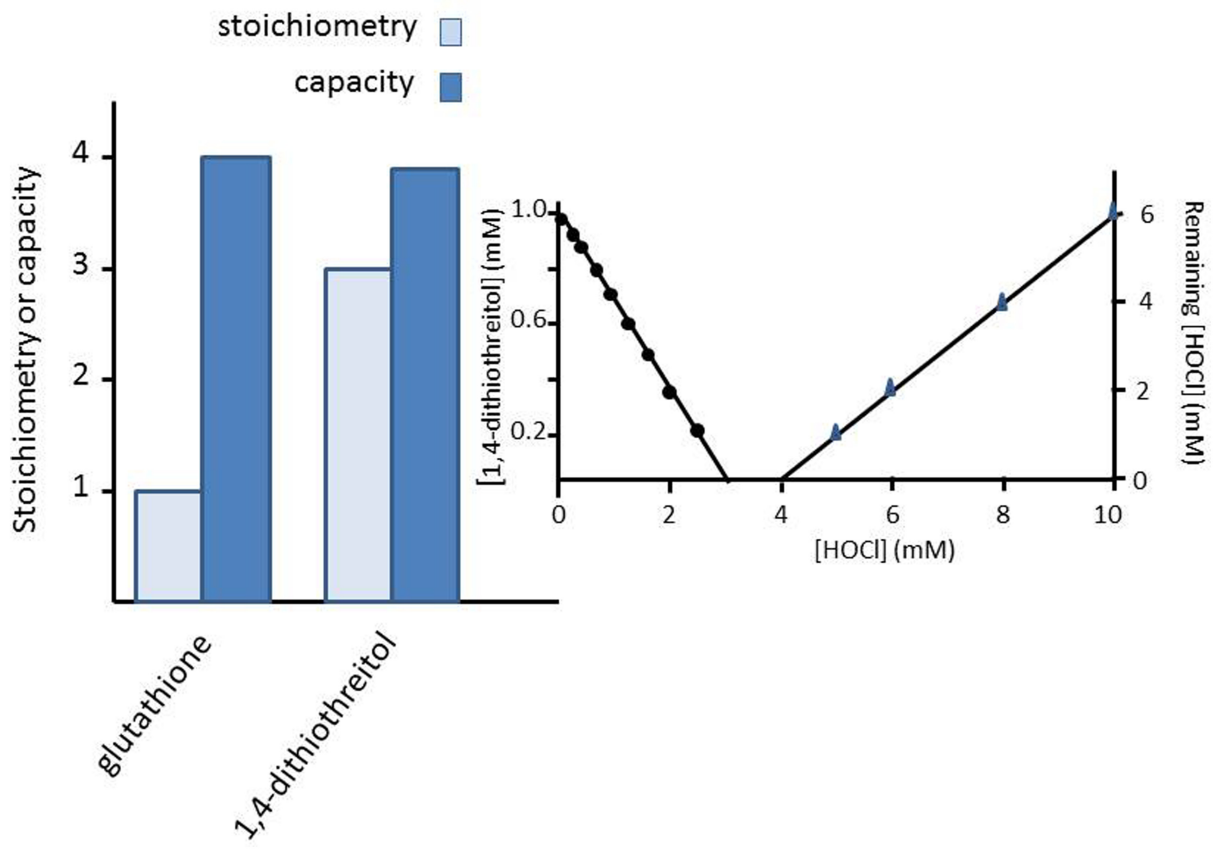

FIGURE 1 | The stoichiometry and the capacity of the reaction of either 1 mM of glutathione (GSH) or 1 mM of 1,4-dithiothreitol with increasing concentrations of HOCl (up to 10 mM). The inset shows the bar diagrams are obtained for 1,4-dithiothreitol. Note that three molecules of 1,4-dithiothreitol are consumed, but only after the consumption of almost four molecules HOCl is detected. This leads to a stoichiometry value of 3 and a capacity of 4. Similarly, GSH has a stoichiometry of 1 and a capacity of 4.

of antioxidant that reduces the signal produced by the detector by 50 percent compared to the signal obtained in the absence of the antioxidant.

The advantage of the HOCl scavenging assay is that all of the reactive species are scavenged by either the detector or antioxidant, and therefore there is no systematic underestimation of antioxidant activity as in numerous other assays.

RESULTS

Glutathione is an important antioxidant and its protective action in various lung diseases is certain. In fact in several large multicenter clinical trials the GSH precursor *N*-acetylcysteine was used in attempts to increase the lung epithelial levels of GSH. For example in COPD patients *N*-acetylcysteine was shown to decrease the intensity and frequency of exacerbations (Decramer et al., 2005). In idiopathic pulmonary fibrosis patients three times 600 mg daily resulted in less worsening of the lung function (Demedts et al., 2005).

Damage to numerous vital enzymes in the lung by oxidants has been associated with various lung diseases. GSH protects. One of the oxidants involved is HOCl. This oxidant is generated from neutrophils by the enzyme myeloperoxidase (Park et al., 2013)

Glutathione is known to react with HOCl. We therefore investigated and re-evaluated GSH in various competition assays to establish its efficacy to protect physiologically critical enzymes in lung pathology which are subjected to the oxidant HOCl.

Acetylcholinesterase is among others of importance in lung physiology, because it removes the smooth muscle contracting acetylcholine. Damage by HOCl to acetylcholinesterase will lead to lung smooth muscle contraction and hyperreactivity by disturbing the cholinergic homeostasis (den Hartog et al., 2002). The EC_{50} value of GSH against damage by 1 μM HOCl on acetylcholinesterase is 0.2 μM (den Hartog et al., 2002).

Alpha-1 antiprotease (or α_1-antitrypsin or protease inhibitor) is another important enzyme in lung pathophysiology since it inactivates elastase. The latter is released during inflammation by neutrophilic granulocytes and breaks down connective tissue fibers elastin. Oxidative damage of alpha-1 antiprotease has been associated with the lung disease emphysema.

In a competition assay in which the protection by GSH is characterized, the EC50 value of GSH against damage by 100 μM HOCl on alpha-1 antiprotease is 20 μM.

The amino acid which is critical in the function of alpha-1 antiprotease is methionine (Taggart et al., 2000). We therefore also investigated the protection of GSH on the HOCl mediated oxidation of methionine. The EC_{50} value of GSH against oxidation by 630 μM HOCl of methionine is 520 μM.

Literature data show that albumin oxidation by 1600 μM HOCl is inhibited by GSH with an EC_{50} value of 200 μM (Gatto et al., 2002). With lower levels of HOCl (200 μM) led to an EC_{50} of GSH of 38 μM (Yan et al., 1996).

In an effort to understand these striking differences in EC50 values of the protective action of GSH on HOCl-induced oxidative damage (as summarized in **Table 1**), we computed the reaction of GSH with HOCl. It seemed that stoichiometrically one molecule of GSH is rapidly consumed by reaction with one molecule HOCl but the total HOCl scavenging capacity of GSH amounts to 4, i.e., four molecules of HOCl are scavenged in total by one molecule of GSH (**Figure 1**).

For comparison, 1,4-dithiothreitol has a stoichiometry of three and a total capacity of almost four (**Figure 1**).

DISCUSSION

Competition experiments are frequently used to establish the protection of antioxidants against oxidative damage of important oxidative sensitive biological targets [detectors, (D)]. The competition experiments are relatively easy to perform and frequently give rise to far reaching conclusions on the value of antioxidants. These seemingly uncomplicated experiments have an outcome that is, however, more complex to interpret.

We already published that the initial concentration of the reactive species (R) can influence the observed activity of an antioxidant (Balk et al., 2009).

It can be derived that the EC_{50} value of antioxidants is linked to the rate constant (k_a) of the antioxidant with the reactive species, as follows:

$$k_a = (k_d/EC_{50})\,[D]_0$$
$$EC_{50} = (k_d/k_a)\,[D]_0.$$

In this derivation it is assumed that the concentration of the detector [D] does not vary during the experiment and equals the initial concentration $[D]_0$, likewise it is assumed that [A] remains constant during the experiment and equals the initial antioxidant concentration $[A]_0$. We published earlier that concentrations of [D] and [A] do not decrease to the same extent in a competition experiment (Balk et al., 2009). This greatly affects the outcome of competition experiments.

The current results show that even under the above strict conditions it is sometimes not just the reaction rate constant k_a of the antioxidant with the oxidant that governs the antioxidant protection of biological targets. This is clearly presented in **Figure 1**. Stoichiometrically, one molecule of GSH rapidly reacts with HOCl, but at the same time in total four molecules of HOCl are scavenged by one molecule of GSH. For comparison, 1,4-dithiothreitol has a stoichiometry of three and has a total capacity of almost four molecules (**Figure 1**).

Table 1 | The antioxidant activity of glutathione (GSH) indicated by its EC_{50} value in various completion assays.

	EC_{50}-value (μM)	[HOCl] (μM)	Reference
Acetylcholine esterase	0.2	1	den Hartog et al. (2002)
Alpha1-antiprotease	20	50	Haenen and Bast (1991)
Methionine	520	630	This paper
Albumin	38	200	Yan et al. (1996)
	200	1600	Gatto et al. (2002)

Protection by GSH on HOCl-induced damage. The initial HOCl concentration is depicted as [HOCl].

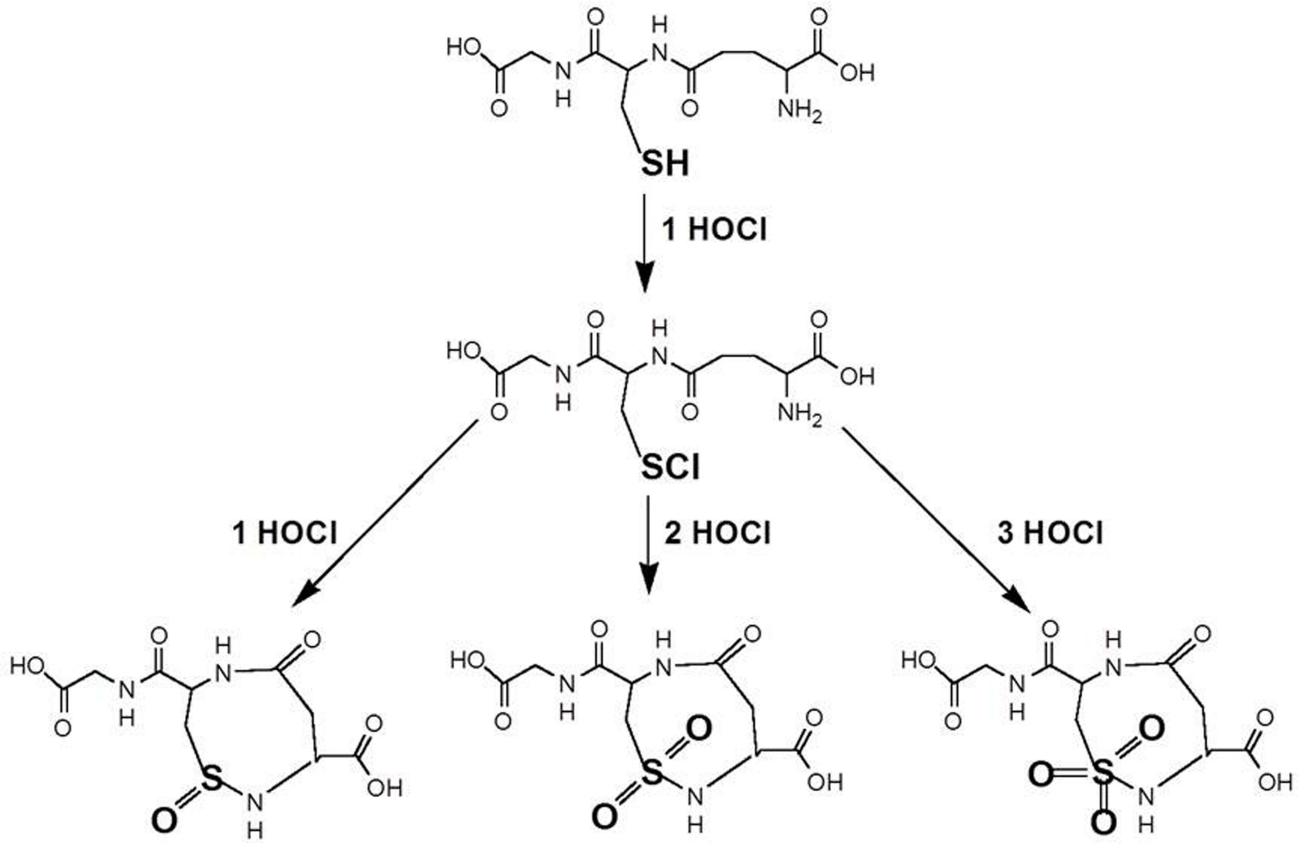

FIGURE 2 | Putative reaction scheme of GSH with OCl^- (after Winterbourn and Brennan, 1997). The initial reaction is the formation of glutathione sulfenyl chloride (GSCl). Further suggested products are depicted as oxidized condensation products of the sulfonyl chloride with the amino group of the glutamyl residue of GSH.

Table 2 | The genuine scavenging activity of glutathione (GSH) which is obtained from by $\{[HOCl]_0/EC_{50}\} \times 1/2$.

	Genuine scavenging activity
Acetylcholine esterase	2.5
Alpha1-antiprotease	1.25
Methionine	0.6
Albumin	2.63
	4

Glutathione has a total HOCl scavenging capacity of four. It appears that stoichiometrically one molecule of GSH is rapidly consumed by reaction with one molecule HOCl but the total HOCl scavenging capacity of GSH amounts to 4, i.e., four molecules of HOCl are scavenged in total by one molecule of GSH (**Figure 1**).

It is suggested that GSH reacts with HOCl in a step wise fashion to form four products. Interestingly, several higher oxidation products have been suggested to occur before (Winterbourn and Brennan, 1997). It might be proposed that the initial reaction is $GSH + OCl^- \rightarrow OH^- + GSCl$ (**Figure 2**). The observed stoichiometry of four indicates that the fourth product has a slower reaction rate with HOCl than GSH. It is not uncommon that oxidation products of antioxidants are more reactive than the parent antioxidant as we have reported earlier (Arts et al., 2004).

Interestingly several other reaction products have indeed been suggested, including a GSH sulfonamide (Pullard et al., 2001; **Figure 2**).

Apparently, the products of the reaction of GSH with HOCl also add to the total antioxidant activity of GSH. The contribution of these higher oxidation products of GSH can be estimated from the observed antioxidant activity.

If one antioxidant molecule would scavenge one HOCl then would the EC_{50} value be half of the initial HOCl concentration ($[HOCl]_0$) and thus would in that case the ratio $[HOCl]_0/EC_{50}$ be 2. In other words $\{[HOCl]_0/EC_{50}\} \times 1/2$ will be 1.

In the scenario that the oxidation products of the antioxidant contribute to the antioxidant activity the $\{[HOCl]_0/EC_{50}\} \times 1/2$ will be higher than 1. This ratio has been calculated (**Table 2**) for GSH protection of HOCl-induced damage of acetylcholine esterase (2.5), alpha-1 antiprotease (1.25), methionine (0.6), and albumin (4). The ratio $\{[HOCl]_0/EC_{50}\} \times 1/2$ gives the average number of HOCl molecules scavenged by the antioxidant GSH at EC50 conditions. Comparing the extremes (methionine versus albumin as target or detector) shows that there is a large difference

in the contribution of the oxidation products of GSH in the overall protection against the HOCl-induced damage to either methionine or albumin by GSH. This can be explained by the difference in reaction rate between HOCl with methionine (which is relatively high) and the rate between HOCl with albumin (which is relatively low).

Also in the protection by GSH against HOCl induced damage to acetylcholine esterase or alpha-1-antiprotease, the reaction products of GSH contribute to the overall activity of GSH.

Evidently, the reaction rate constant k_a determines whether scavenging occurs. However, the genuine scavenging activity is primarily determined by the number of oxidant molecules (in this study HOCl) scavenged. GSH is not just a good biological antioxidant because of its reaction rate with HOCl which is generated during inflammations by myeloperoxidase but rather because the higher oxidation products generated are effective antioxidants. The amount of reactive HOCl molecules scavenged by GSH rather than its initial reaction rate marks GSH a better antioxidant than previously thought!

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Potential role of glutathione in evolution of thiol-based redox signaling sites in proteins

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Cysteine is susceptible to a variety of modifications by reactive oxygen and nitrogen oxide species, including glutathionylation; and when two cysteines are involved, disulfide formation. Glutathione-cysteine adducts may be removed from proteins by glutaredoxin, whereas disulfides may be reduced by thioredoxin. Glutaredoxin is homologous to the disulfide-reducing thioredoxin and shares similar binding modes of the protein substrate. The evolution of these systems is not well characterized. When a single Cys is present in a protein, conjugation of the redox buffer glutathione may induce conformational changes, resulting in a simple redox switch that effects a signaling cascade. If a second cysteine is introduced into the sequence, the potential for disulfide formation exists. In favorable protein contexts, a bistable redox switch may be formed. Because of glutaredoxin's similarities to thioredoxin, the mutated protein may be immediately exapted into the thioredoxin-dependent redox cycle upon addition of the second cysteine. Here we searched for examples of protein substrates where the number of redox-active cysteine residues has changed throughout evolution. We focused on cross-strand disulfides (CSDs), the most common type of forbidden disulfide. We searched for proteins where the CSD is present, absent and also found as a single cysteine in protein orthologs. Three different proteins were selected for detailed study—CD4, ERO1, and AKT. We created phylogenetic trees, examining when the CSD residues were mutated during protein evolution. We posit that the primordial cysteine is likely to be the cysteine of the CSD which undergoes nucleophilic attack by thioredoxin. Thus, a redox-active disulfide may be introduced into a protein structure by stepwise mutation of two residues in the native sequence to Cys. By extension, evolutionary acquisition of structural disulfides in proteins can potentially occur via transition through a redox-active disulfide state.

Keywords: cross-strand disulfide, forbidden disulfide, redox-active disulfide, exaptation, disulfide evolution, CD4 evolution, AKT evolution, post-translational cysteine modification

INTRODUCTION

Thiol-based redox signaling is the collective name for biochemical pathways that regulate cellular processes by post-translational modification of sulfur moieties in cysteine (Cys) and methionine (Met) residues of proteins. These pathways are pathologically dysregulated in diseases of oxidative stress which include cancer, neurodegenerative diseases, heart disease and aging. A better understanding of these pathways is essential to diagnosis, treatment, and prevention of these diseases.

Cys residues are susceptible to a variety of modifications by reactive oxygen and nitrogen oxide species (ROS, RNS). Cys can be nitrosated, glutathionylated, and can form covalent bonds with other Cys. RNS such as nitric oxide (•NO) can mediate S-nitrosation to yield an S-nitrosothiol (RSNO). Other RNS, such as peroxynitrite ($ONOO^-$), can also mediate S-nitration to yield S-nitrothiols ($RSNO_2$). Sequential oxidation of Cys thiols yields sulfenic (–SOH), sulfinic ($–SO_2H$), or sulfonic ($–SO_3H$) acid derivatives. Reaction of protein thiols with low-molecular weight thiols such as glutathione (GSH) can yield mixed disulfides. Alternatively, oxidation by ROS or RNS can result in a disulfide bridge forming between two thiols, either within a protein chain or between protein chains (Wouters et al., 2011).

Reduction of these systems is effected in a variety of ways. For some disulfides, autoreduction within a protein may be assisted by mechanical stresses and the local electrostatic environment. For example, arsenate reductase (ArsC), an enzyme involved in detoxification of arsenic, transfers electrons through a series of Cys modifications, manifested largely as disulfide isomerizations, as part of its catalytic cycle (Messens et al., 2002). Two of the three disulfides formed in the oxidized states of ArsC are autoreduced, while the disulfide in the final oxidized state is reduced by thioredoxin (Trx) (Messens et al., 2002; Wouters et al., 2010). In general,

reduction of surface accessible disulfides may be effected by the GSH redox buffer (Ostergaard et al., 2001), or specific thiol oxidoreductases such as Trx. GSH-Cys adducts may be reduced by glutaredoxins (Grxs), which are homologs to Trxs (Martin, 1995), and share similar binding modes of the protein substrate with Trxs.

It is now understood that a continuum of redox set points within proteins control cellular processes in response to a range of ROS fluxes. Low fluxes mediate homeostatic control of housekeeping redox processes, while higher fluxes mount stress and adaptive responses before a threshold is reached, triggering apoptosis, or programmed cell death. Partial loss of control of these processes is now believed to underlie aging (Humphries et al., 2006). Even higher levels of ROS can cause cellular necrosis.

In addition to its roles in housekeeping and the stress response, thiol-based redox regulation is also important in developmental processes in plants and animals. In plants, thiol-based redox regulation is involved in seed germination (Buchanan, 1980). In animals, the early embryo forms under reducing conditions (Hartley, 1960), but the embryonic environment becomes more oxidizing as the embryo grows (Hartley, 1960; Ibrahim et al., 2006). Redox-regulated processes govern the formation of substructures during embryo development by selective apoptosis.

The evolution of these systems is not well characterized. Among prokaryotes, the well-studied GSH system of eukaryotic redox regulation operates only in purple bacteria and cyanobacteria (Fahey, 2001; Mallick et al., 2002). In other prokaryotes, different redox buffers are utilized, for example acetyl-coenzyme A in gram positive bacteria (Hummel et al., 2005). It appears that the role of GSH as a redox buffer is predated by its role in detoxification. Glutathione S-transferases (GSTs) label xenobiotics with GSH, allowing them to be removed from the cell by specific transporters. GSTs are found in all classes of eukaryota and bacteria. Within GSTs, the GSH-conjugating site lies in a Trx-like domain (Atkinson et al., 2009). These data suggest that a common stress-related ancestor with a Trx-like fold may have pre-dated the evolution of the thiol-oxidase function now most commonly associated with the Trx-like fold.

In its role as a redox buffer, GSH is conjugated to reactive Cys of endogenous proteins, inducing conformational changes in the substrate proteins, and effecting a signaling cascade that evokes biological responses (Wouters et al., 2011). Conformational changes are generally small, involve the protein backbone, and are often accompanied by a local increase in protein disorder (Mallis et al., 2000; Wouters et al., 2011). Surface modification of proteins such as carbonic anhydrase by GSH results in significant disorder of the GSH distal to the covalent bond (Mallis et al., 2000). In this disordered state, GSH may act as a flipper, disrupting protein interactions. Thus, introduction of a single Cys into a protein may allow reversible GSH conjugation to occur, effectively introducing a simple redox switch into the protein.

If a second Cys is introduced into the protein sequence, the potential for disulfide formation exists. In favorable configurations and protein contexts, a bistable redox switch may be formed (Wouters et al., 2010). This has the potential to effect two different "active" states of the protein. For example, the protein may interact with certain protein partners with the switch in one redox state, and with other protein partners when the switch is in the alternate redox state.

How are these redox-regulated sites introduced into proteins? The introduction of potential disulfide-forming thiol pairs may be facilitated by the fact that both Cys do not need to be introduced into the protein chain simultaneously. This may seem obvious in isolation but, *in toto*, a number of constraints must be satisfied, and the likelihood of this occurring is greatly increased if the process can occur in a stepwise manner. Incorporation of a single Cys may make the protein immediately responsive to a range of oxidative modifications, including nitrosation and glutathionylation (**Figure 1**). In order for these modifications to be effective, the context of the Cys within the protein must be suitable. To be modifiable, the Cys must have a low pKa, a property that is largely determined by the electrostatic environment of the protein context. In addition, in order for GSH to be reductively removed from the Cys, Grx must be able to dock to the backbone near the modified Cys. Thus, there are additional requirements on the secondary structure and accessibility of the introduced Cys residue. Introduction of a second Cys at a later stage may then enable disulfide formation subject to further constraints. Because Grxs are homologs to Trxs and share similar binding modes of the protein substrate, the complete redox cycle may potentially be immediately effected. So the similarity in the constraints of 1-Cys and 2-Cys reduction facilitates this second Cys acquisition step, but an additional constraint is that the second Cys must be the resolving Cys and thus have a higher pKa than the existing Cys.

Anecdotally, a number of these 1-Cys/2-Cys homologs have been described (Wouters et al., 2010). An example where one protein is glutathionylated and a homolog forms disulfides is the Grx–Trx pair itself. Trx forms a disulfide between the Cys residues of the CXXC motif as part of its reaction cycle. Grx contains only the reactive N-terminal Cys of the CXXC motif which is glutathionylated during its reaction cycle. Another pair of families is the 1-Cys and 2-Cys peroxiredoxins (Prxs) (Copley et al., 2004). When the resolving Cys is present, Prxs are reduced by Trx. When it is not, they are reduced by Grx. In all the above examples, the additional Cys is the resolving Cys, suggesting the reactive Cys must come first in order for the mutation to become fixed in the population. These examples demonstrate evolutionary exaptation from the Grx pathway to the Trx pathway, or vice versa, does occur; and likely can be easily effected for other proteins.

The vast database of sequenced genomes of organisms is a valuable resource which can be mined for information regarding the evolution of these pathways, leading to a better understanding of their function and control. Here we searched for examples of protein substrates where the number of redox-active Cys residues has changed throughout evolution.

We focused on cross-strand disulfides (CSDs), a type of forbidden disulfide motif. Forbidden disulfides are a group of canonical disulfides that disobey elucidated rules of protein stereochemistry (Wouters et al., 2010). CSDs are metastable disulfides: their intermediate disulfide torsional energies render than more easily reduced than low energy structural disulfides, but they are more stable than high energy disulfides (Wouters et al., 2007, 2011). Their reduction is likely assisted by endogenous proteins. The postulated role of CSDs as canonical redox switches is supported

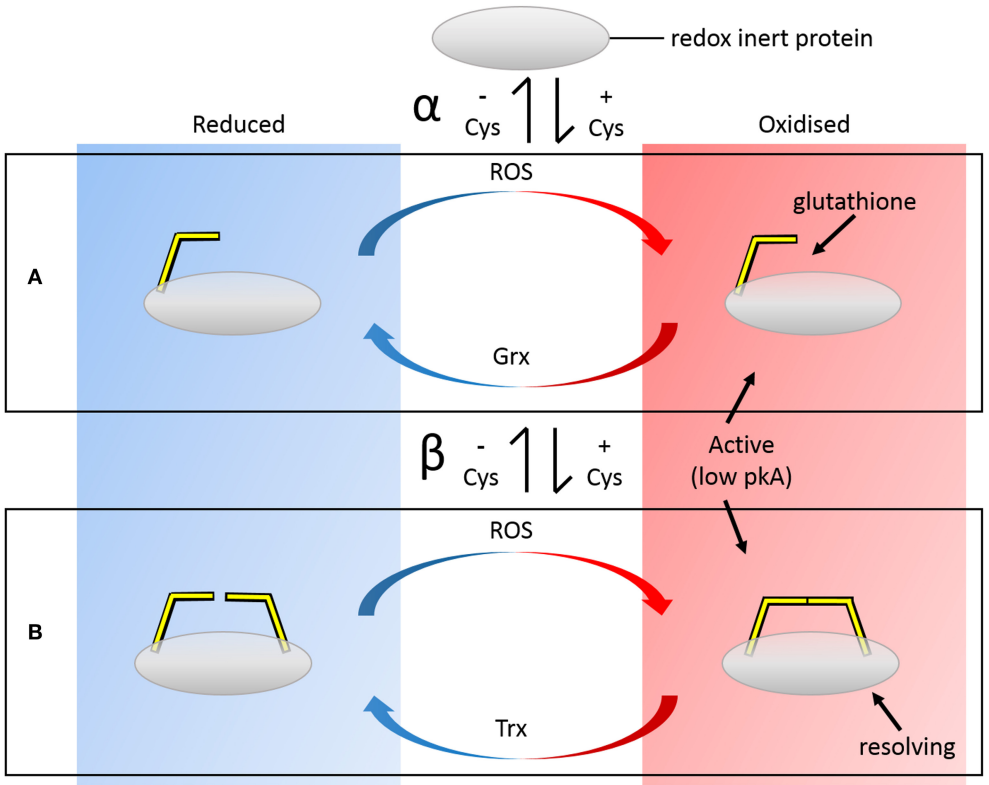

FIGURE 1 | Panels depict biochemical redox cycles of (A) single Cys and (B) disulfide-forming pair of Cys residues and their evolution (white central vertical panel). Incorporation of a single Cys (α) may make the protein immediately responsive to a range of oxidative modifications, including nitrosation and glutathionylation. Cys with lower pKas are more susceptible to modifications. Introduction of a second Cys (β) at a later stage may then enable disulfide formation, preventing overoxidation of the low pKA "active" Cys and effectively forming a bistable redox switch. Because Grxs are homologs to Trxs and share similar binding modes of the protein substrate, the complete redox cycle may potentially be immediately effected. These evolutionary modifications are reversible i.e., the resolving Cys may be lost, returning the protein to the Grx pathway etc.

by anecdotal information underpinning a functional redox role in specific examples (Haworth and Wouters, 2013). In particular, several CSDs are known Trx substrates (Maeda et al., 2005), and we have postulated recently that CSDs are, in general, cognate substrates of Trx (Haworth and Wouters, 2014).

Here, we focused specifically on protein orthologs where the CSD is present, absent and also found as a single Cys. We provide a number of examples and speculate on the evolutionary advantages of the changes.

MATERIALS AND METHODS

We mined the Protein databank (PDB) (Berman et al., 2000) for proteins with CSDs, the most common type of forbidden disulfide. In a dataset of 29,261 disulfide-containing proteins, 195 unique protein clusters containing 235 CSDs were retrieved (Haworth and Wouters, 2013). These proteins were mapped to Uniprot (Magrane and Consortium, 2011) using the PDB identifiers. For each protein sequence in this CSD set, sequences in the corresponding 50% cluster of homologs from Uniprot were retrieved and aligned.

SELECTION OF PROTEINS FOR STUDY

The proteins used in this study were chosen from the above list based on two criteria—firstly, that there was good sequence evidence (i.e. more than one ortholog) that an intermediate 1-Cys state for the protein existed, and was retained in some species; and secondly, that there was evidence that the protein was redox regulated. From this set of CSD-containing proteins, three different proteins were selected for detailed study—CD4, ERO1, and AKT1. A fourth protein, DAPP1, satisfied the first criterion, but because we could find no evidence supporting its redox activity, it was not considered further. The corresponding identifiers for the selected proteins in Uniprot were P01730 (CD4_HUMAN), Q96HE7 (ERO1A_HUMAN), and P31749 (AKT1_HUMAN). Data for the corresponding protein structures 4h8w, 3ahq, and 1unr was retrieved from PDBsum (De Beer et al., 2014).

MULTIPLE SEQUENCE ALIGNMENT AND TREE GENERATION

For each seed sequence, an initial alignment was performed in Uniprot using the sequences in the 50% sequence identity cluster (Clusterref_50) with the "Align" option. The raw multiple sequence alignment from Uniprot was loaded into Jalview (2.8.1) (Waterhouse et al., 2009) for editing, visualization and analysis. Masking was performed to remove ambiguous regions of the alignment, and the initial tree was generated with the Neighbor-joining method using the BLOSUM 62 substitution matrix. Additional sequences were added to the alignment using a combination of Blast searches (Altschul et al., 1990) of Uniprot,

and joining of additional 50% sequence identity (ID) clusters to provide further evolutionary depth when necessary. The generated tree in Newick format was loaded into the evolutionary tree builder MEGA6 (Tamura et al., 2013) and the root chosen using an outgroup from the accepted species tree (Murphy and Eizirik, 2009). The figure for the final tree was created in MEGA6 and further manipulated in Illustrator 6 (Adobe Systems). The number of Cys residues in the CSD motif was mapped onto the phylograms manually.

CD4

For CD4, the human cluster Uniref50_P01730 containing 42 sequences from 20 different species was initially retrieved. Multiple sequences were excluded: including 15 partial sequences, seven Refseq sequences, an uncharacterized protein, a testis cDNA sequence and two surface antigen sequences; resulting in an initial alignment built on 16 sequences from seven different species. Since the sequences in the cluster did not provide enough evolutionary information, we extended our analysis using a Blast search (Altschul et al., 1990) in Uniprot using P01730 as a template. The results from the Blast search were again filtered, excluding alternate isoforms from the same species, resulting in an additional 125 CD4-like sequences being added to the alignment. Masking was performed to remove alignment ambiguities near gaps. As the alignment was ambiguous in the region of the CSD in fishes, turtle, duck, snakes, and frogs, these sequences were excluded from further consideration. The final tree was built on an alignment of 51 sequences from 50 different species using residues homologous to 85–129, 150–230, 233, 236–244, 281–302, 307–348, and 351–373 of the human sequence. The disulfide of interest forms between Cys residues at positions 154 and 184 of the human sequence P01730 (130 and 159 in the PDB structure: 4h8w). The complete phylogram is shown in Supplementary Figure 1. Further analysis of sequence conservation was performed with Jalview on sequence subsets. In one subset, the sequences of 16 primate and three muroid sequences, where the disulfide was conserved, were analyzed separately in the vicinity of the CSD. In two additional subsets, separate pairwise alignments of human and galago CD4, and mouse and Chinese hamster CD4 were performed as a part of more detailed analyses (Supplementary Figure 2).

ERO1

For ERO1, the cluster Uniref50_Q96HE7 built on the human sequence, containing 66 protein sequences from 37 species, was used in the initial alignment. Most of the sequences were ERO1A and ERO1B genes in higher animals. In order to retrieve more evolutionary information, a Blast search was performed in Uniprot using the human ERO1A protein Q96HE7 as the template. This retrieved ERO1-like sequences from divergent species including worms, beetles, lancelet, fishes, fruit flies, and mosquitoes. The 50% identity cluster Uniref50_L7MC09 built on the *Rhipicephalus pulchellus* sequence, with sequences from lancelet (C3Z2H2), red flour beetle and polychaete worms, was incorporated, and an additional Blast search using the ERO1 protein of *Capitella teleta* (Polychaete worm) was performed. The resulting alignment of 177 sequences was further refined by choosing a representative sequence for branches with very small branch lengths. Masking was performed and a tree built from the final alignment containing 119 sequences using regions 33–58, 61–103, 108–115, 137–144, 157–160, 174–210, 239–270, 275–287, 295–324, 328–335, 338–363, 371–386, 390–393, 402–418, 433–438, and 444–456 homologous to the human ERO1. A Newick tree was generated in Jalview and loaded into MEGA6, where the Black flying fox sequence (Uniprot id: L5K102) was chosen as the root. The two CSDs of interest are nested in the sequence at positions 35 and 48 and 37 and 46 (PDB: 3ahq and Uniprot: Q96HE7). A complete phylogram is shown in Supplementary Figure 3.

AKT

For AKT, the Uniref50_P31751 cluster was retrieved with 121 sequences from 69 organisms. Of these, 54 sequences from 38 different organisms, from human to snake, were mapped to Uniprot and the initial alignment was built. This was merged with UniRef50_Q17941, a 50% sequence ID cluster built on *Caenorhabditis elegans* AKT1 (Q17491) which mapped to 19 Uniprot sequences from 18 different organisms, mostly arthropods; and the 50% cluster Uniref50_Q9XTG7 built on *C. elegans* AKT2 with nine mapped sequences from sea squirt, *C. vulgaris* and platyfish. A Blast search was performed with the Honeybee AKT1 (H9KF44) as the template. After removing the duplicates, the resulting sequences were aligned in Uniprot and merged with the previous alignment. After masking, the final alignment was built with 262 sequences with residues 28–45, 48–71, 76–113, 146–210, 215, 230–266, 269–302, and 335–427 homologous to the human AKT1 protein P31749. A Newick tree was generated and loaded into MEGA6. The land crab sequence was chosen as the root. The disulfide of interest is formed between Cys 60 and Cys 77 of the human AKT1 protein (PDB: 1unr and Uniprot: P31749). The complete phylogram is shown in Supplementary Figure 4.

PHYSICOCHEMICAL PROPERTIES OF CSDs

To further investigate the lability of the CSDs of interest we calculated the torsional energies of the disulfide bonds; and, where possible, the pKas of the two involved Cys. Torsional energy calculations were performed using an online torsional energy calculator[1] based on input dihedrals, which uses a combined quantum chemical (χ_2, χ_3, χ_2') and empirical calculation (χ_1, χ_1') described in detail elsewhere (Haworth et al., 2010). Dihedral angles were calculated using Pymol[2]. Calculations of pKa for individual Cys residues were performed with Propka[3] which gives an approximation of the pKa based on a solution of the Boltzmann equation (Rostkowski et al., 2011). The only reduced structure available was for AKT (PDB: 1unq).

RESULTS

We previously mined the PDB for proteins with CSDs, the most common type of forbidden disulfide (Haworth and Wouters, 2014). Forbidden disulfides are strained disulfides that occupy

[1]http://www.sbinf.org/applications/pes.html
[2]http://www.pymol.org/
[3]http://propka.org

recognizable contexts in primary and secondary structure of proteins. The contexts were originally declared "forbidden" by analyses that considered the geometric constraints of disulfide formation and hydrogen bond formation in secondary structure (Richardson, 1981; Thornton, 1981). Later work has shown that disulfides in these strained contexts do occur, despite their strain, which likely relates to their functional redox role in proteins (Wouters et al., 2010; Haworth and Wouters, 2013). The frequency of CSDs in solved protein structures is increasing, likely due to better control of redox conditions during structure solution (Wouters et al., 2010). Because CSDs can act as redox switches, failure to control conditions carefully results in the proteins adopting different structural states. This heterogeneity adversely affects the crystallization process.

CSDs have been introduced into protein sequences extensively throughout evolution, especially in molecules of the immune system, particularly the adaptive immune system (Wouters et al., 2007). In order to study this process in the selected proteins of interest, we scanned homologs of proteins with CSDs for sets where the disulfide was not completely conserved. Three proteins were chosen where a CSD has been introduced relatively recently in evolutionary history; and where the 1-Cys evolutionary intermediate state was preserved in some species: CD4, ERO1, and AKT. For each of these proteins, alignments of the homologs were created from Uniprot clusters and Blast searches. Alignments were masked and evolutionary trees created as described in detail in the methods.

CD4

CD4 is a critical protein of the adaptive immune system, which may be expressed on the surface of some lymphocytes, including T helper cells, monocytes, macrophages, and dendritic cells. CD4 T helper cells signal to other immune cells, such as CD8 killer cells, to alert them to the presence of infectious agents which must be targeted. CD4 is a co-receptor for major histocompatibility complex (MHC) class II molecules, and the primary cell surface receptor for Simian immunodeficiency virus (SIV)/Human immunodeficiency virus (HIV) (Dalgleish et al., 1984).

In CD4+ T cell antigen receptor-mediated signaling, the polymorphic regions of the MHC class II proteins engage the receptor complex, whereas the non-polymorphic regions engage the CD4 co-receptor (Parnes, 1989). The TCR/MHCII/CD4 supercomplex then recruits intracellular molecules such as LCK to effect signaling.

The CD4 molecule is a single-pass transmembrane protein with 4 extracellular immunoglobulin (Ig) domains and a short cytoplasmic tail. The Ig domains comprise a duplication of a pair of Ig variable (IgV) and Ig constant (IgC) domains arranged in tandem (**Figure 2B**). Structurally, IgV domains are slightly larger than IgC domains, having more strand elaborations on the basic Ig domain fold (Halaby et al., 1999). Ig domains are extremely common domains that are frequently involved in redox regulation (Wouters et al., 2010; Haworth and Wouters, 2013). Ig domains and their antecedents, the fibronectin type III (FIII) domains are found in many proteins of both the innate and adaptive immune system including cytokine receptors (FIII domains); and Ig domain-containing MHC homologs such as MICA and RAE-Iβ (Wouters et al., 2007). Ig domains consist of two β sheets that form a sandwich, typically containing seven to nine strands. The seven-stranded variety is labeled from A to G. Additional strands in larger Ig domains (such as the variable domains) are typically found between the C and D strands and are labeled C′, C″ etc. In general, Ig domains are distinguished from FIII domains by an intersheet disulfide that links strand B on one sheet to strand F on the other.

Three regions of CD4 have been implicated to date in important protein-protein interactions (**Figure 2B**). The region of human CD4 that interacts with MHC class II lies mainly within the C″ and D strands of domain 1 (D1), the N-terminal IgV domain (Wang et al., 2001). A second region, located in the short cytoplasmic tail, is a CXC motif that mediates the interaction with lymphoid cell kinase (LCK or p56LCK) necessary for T cell activation (Laing et al., 2006). The third region, which contains the CSD of CD4, is located in D2, the N-terminal IgC domain, joining two β-hairpins. The CSD is formed by a disulfide bond between Cys 130 and Cys 159 in the structure (PDB: 4h8w).

The CSD has been demonstrated to be reduced during the process of T-cell signaling, and its reduction is also co-opted during entry of the HIV viral envelope glycoprotein (gp120) into the cell. During T-cell signaling, it must be reduced in order for the TCR/MHCII/CD4 receptor complex to form: a process that requires CD4 dimerization and domain swapping (Cerutti et al., 2014). Reduction of the CD4 CSD is mediated by Trx, Grx1, and PDI *in vitro* (Gallina et al., 2002; Matthias et al., 2002; Auwerx et al., 2009). Further investigation of the role of the CSD of CD4 in T-cell signaling has recently been performed using four Cys mutants: C159A, a mutant of the putative active Cys of the CSD; C16A/C84A, a double mutant deleting the disulfide from D1; C130A/C159A, a CSD deletant; and C16A/C84A/C130A/C159A, a D1 disulfide and CSD deletant (Cerutti et al., 2014). Experiments with these mutants demonstrated that CD4 binds gp120 of HIV as a reduced monomer; and that CD4 requires reduction of either the D1 or the D2 disulfide to bind gp120, but not both (Cerutti et al., 2014). A redox potential of -241 mV has been determined for the CSD by titration against DTT (Matthias et al., 2010). The higher efficiency of Trx reduction of the CSD suggests the reduction is likely performed by Trx *in vivo* (Cerutti et al., 2014).

The phylogenetic tree for CD4, built from 51 sequences from 50 species is shown in **Figure 2A**. CD4 is thought to have arisen relatively recently in teleost fish following duplication of a two Ig domain-containing ancestral gene, CD4REC (Laing et al., 2006). In trout, CD4, CD4REC and the related protein lymphocyte-activation gene 3 (LAG3) share many similar features (**Figure 2B**) (Laing et al., 2006). The CSD is not present in D2 of trout CD4 which instead contains a disulfide between strands B and F (Laing et al., 2006), typical of most Ig domains (Halaby et al., 1999). The full alignment shows that between the divergence of bony fish and chicken, the Cys on strand B was lost by mutation. The homologous residue is a leucine (Leu) (143 in P01730 and 118 in PDB: 4h8w) in human CD4, and a single unpaired Cys residue remains on strand F in D2 of the chicken sequence. It seems likely this single Cys is a glutathionylation site in chicken. Based on the phylogenetic tree, the acquisition of the second

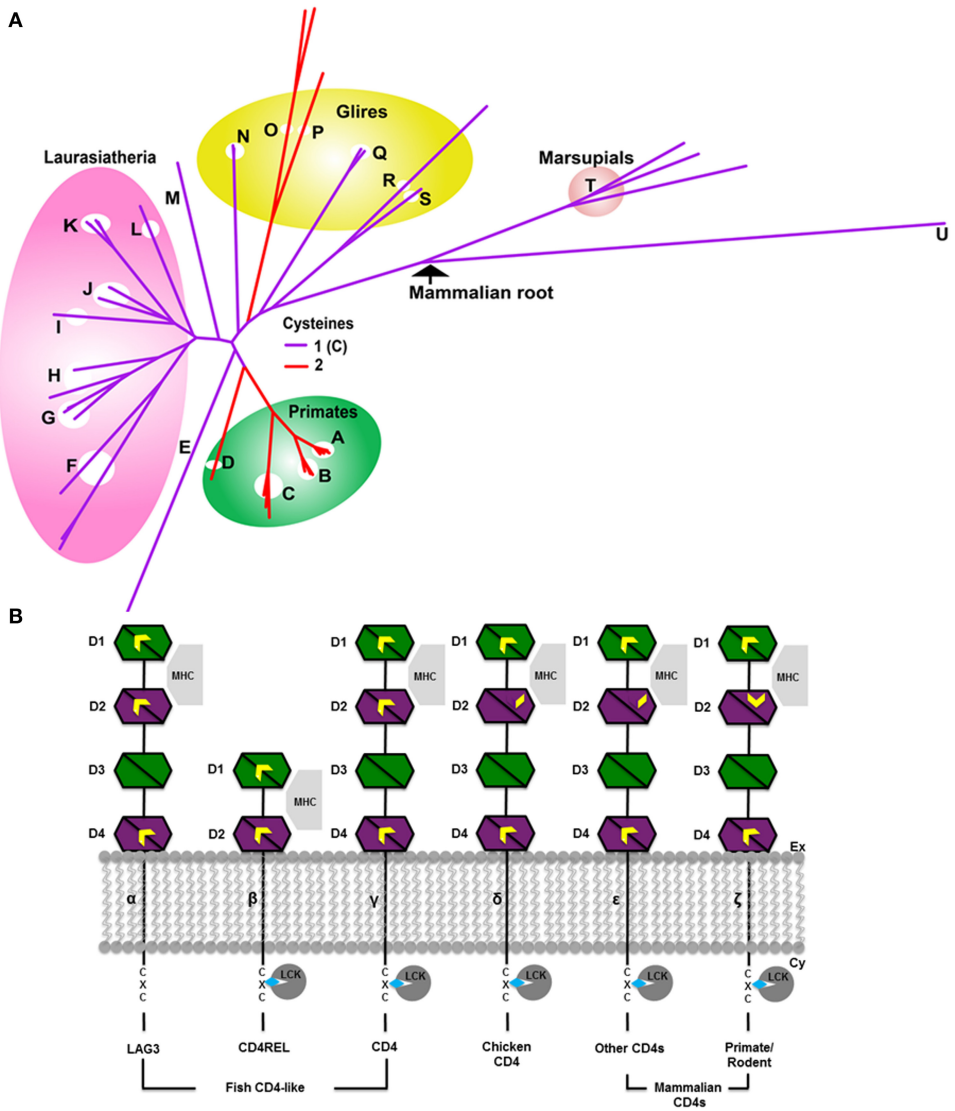

FIGURE 2 | Evolution of CD4 (A) phylogenetic tree in mammals. The color of the branches indicates the number of Cys of the CSD motif in D2. The CSD is found in species with red branches. Species with purple branches have only the C-terminal Cys. The CSD was likely acquired in primates (A–D) around 75 MYA, and independently in muroid rodents *viz* mice, rats (O) and hamsters (P), after the divergence of squirrels (Q, sciuridae), also around 75 MYA. Acquisition of the CSD in muroids was accompanied by rapid evolution of the CD4 protein as evidenced by the long branch lengths of this clade (upper red branches). The four major clades of Eutherian mammals are Afrotheria (including elephants E); Xenartha (none depicted); Laurasiatheria (F–L, shown in pink) and Euarchontaglires (primates, A–D, green circle; glires, N–S yellow circle; and treeshrews, M) (Murphy et al., 2001) Key: A, Lesser apes; B, Greater apes; C, Marmoset (Platyrhines); D, Galago; E, African Elephant; F, Bats; G, Panda/Mink/Ferret; H, Dog and cat; I, Pig; J, Dolphin, Whale and Camel; K, Sheep, Bovine, Goat; L, Horse; M, Tree shrew; N, Rabbit; O, Mouse and rat; P, Chinese hamster; Q, Squirrel/wood chuck; R, Guinea pig; S, Mole rat; T, Wallaby and opossum; U, Platypus. **(B)** Evolution of CD4 and related molecules showing important thiol-containing sites. Molecules are depicted from the more ancestral forms of the molecule (leftmost) to more recently evolved forms to the right. The CD4 homologs consist of two to four extracellular Ig domains, a single transmembrane region and a short cytoplasmic tail. Three important thiol-bearing functional sites have been identified: the CXC motif in the cytoplasmic tail, the between-sheet disulfide in D1, and the cross-strand disulfide (CSD) in D2. The CXC motif is present in all CD4 orthologs **(β–ε)**, but not in the CD4-like LAG3 molecule. The between-sheet disulfide in D1 is present in CD4 and LAG3 **(α–ζ)**. In some teleosts, such as trout and fugu, the Cys on strand B is lost, leaving a single unpaired Cys on strand F. D1 can be definitively identified in CD4REL because it is distinctively encoded on two exons in molecules **β–ε** (Laing et al., 2006). It is involved in interaction with MHC II molecules. Thiols in the D2 site have evolved over time. Originally D2 contained Cys residues on strands B and F, typical of Ig domains (**α–γ**). Around 250 MYA, the thiol on strand B was lost (**δ–ε**). Around 75 MYA, a new thiol appeared on strand C allowing the novel CSD to form upon oxidation (**ζ**). **α–γ** are CD4-like proteins which evolved in a trout ancestor around 450 MYA. Interacting proteins are shown in gray: all proteins bind MHC class II; the CD4-related molecule LAG3 (**α**) binds MHC but not LCK. D3 and D4 contain glycosylation sites which may serve to fend off adventitious interactions with other membrane proteins (Laing et al., 2006). Ig domains are depicted as bisected hexagons with each half representing one sheet: V-like domains are depicted in green; C-like domains in magenta. A CXXC motif in LCK binds to the CXC motif of CD4 via Zn^{2+} (depicted as a blue diamond) (Lin et al., 1998). The extracellular and cytoplasmic sides of the membrane are labeled on the right.

Cys on strand C enabling CSD formation likely occurred independently in two different clades, both within euarchontaglires, one of four large clades of placental mammals (Murphy and Eizirik, 2009). The tree in **Figure 2A** shows only mammals. The CSD is found in primates, which emerged around 75 million years ago (MYA); and was acquired independently in muroid rodents (mice, rats, and hamsters), after the divergence of squirrels (sciuridae), also around 75 MYA (Murphy and Eizirik, 2009). Acquisition of the CSD in both clades was accompanied by rapid evolution of the sequence between the two Cys residues in D2, as shown in the partial alignment in **Figure 3** and in the full pairwise sequence alignments in the supplementary data (Figures S2b, S2c). The pairwise identity for the entire CD4 molecule between human and galago is 74%, and between rat and chinese hamster is 66%; but the pairwise sequence identity over a 52-residue window surrounding the CSD is 63% for the pairwise primate sequences, and 44% for the pairwise muroid sequences. This demonstrates that rapid remodeling of the sequence surrounding the CSD has occurred during the last 75 MYA in both the primate and muroid clades. See further notes on this event in the Discussion.

ERO1A (ENDOPLASMIC RETICULUM OXIDASE)

ERO1 is an essential oxidoreductase involved in disulfide formation of nascent proteins in the endoplasmic reticulum (ER). It acts by reoxidising protein disulfide isomerase (PDI), the enzyme catalyzing disulfide formation. It has been extensively studied in yeast (Frand and Kaiser, 1998). The protein contains multiple redox-active disulfide bonds. In human ERO1, two disulfide-forming Cys pairs, Cys 94/Cys 99 and Cys 394/Cys 397, constitute the redox-active enzymatic center. The first pair of thiols are regulated via isomerisation with a second pair of redox-active thiols to form a pair of nested disulfides between non-consecutive Cys in the sequence (Cys 99–Cys 104, Cys 94–Cys 131) (Hansen et al., 2012).

Near the N-terminus of the sequence, two additional disulfides which form a nested pair, are the CSDs present in the structure of human ERO1 (**Figure 4B**). In the human sequence, the two CSDs are formed between Cys 35 & Cys 48 and Cys 37 & Cys 46. These disulfides are deemed to have a "structural role" because they do not appear to be involved in thiol-disulfide exchange with the enzymatic center (Inaba et al., 2010), nor are they conserved across all ERO1 genes. However, the identity of these two disulfides as CSDs suggests a redox role, possibly as substrates of a Trx-like enzyme (Haworth and Wouters, 2013). The role of the Cys residues of the N-terminal thiol region (NTR) has not been investigated by mutation but constructs of human ERO1A and yeast ERO1 lacking the NTR are often used for functional assays (Cabibbo et al., 2000; Sevier et al., 2007; Hansen et al., 2012). This region may have a regulatory role.

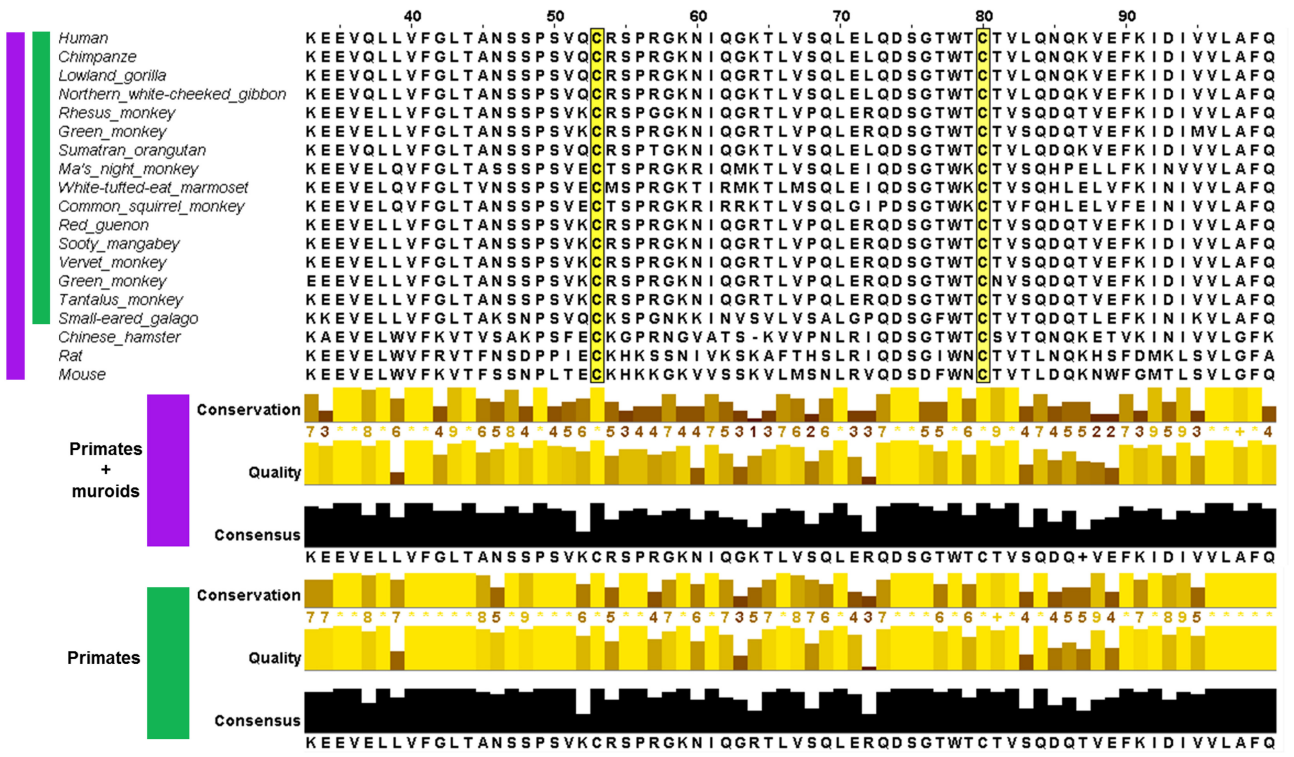

FIGURE 3 | Multiple sequence alignment of CD4 in the region of the CSD in species where both Cys are conserved (upper panel) and measures of its conservation (lower panel) across two groups: primates and muroids (purple), and primates only (green). For each group, the conservation of the amino acid residue in each column, quality (BLOSUM62 score based on observed substitutions) and consensus (most common residue and its proportion for each column) as determined by Jalview (Waterhouse et al., 2009) is shown. The sequence alignment of CD4 showed less conservation in the vicinity of the CSD motif. In the portion depicted, the average conservation across the primate + muroid lineages is 6.2 for residues 53–80 (CSD region highlighted in yellow) and 7.9 within the primate group.

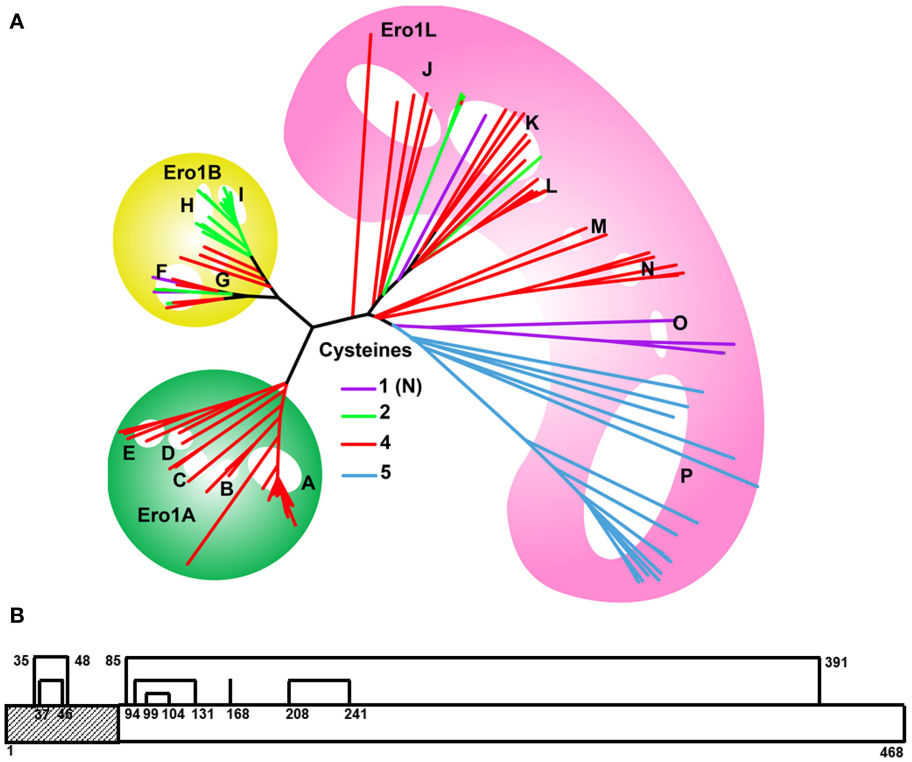

FIGURE 4 | (A) Evolution of the ERO1 gene family. Branches are colored according to the number of Cys present in the NTR of each species, as shown in the legend. A single ERO1-like ancestral gene (pink background) is present in eukaryotes from yeast to lamprey (J–P). With the exception of plants (P), the majority of these genes have four Cys in the NTR (red branches). ERO1 duplicated prior to the divergence of bony fishes. ERO1A (green background) genes retained the nested Cys motif, as does ERO1B (yellow background) in early diverging species such as fish, sharks, and amphibians (F, G). However, later diverging species such as birds, reptiles, and mammals (H, I) have only two Cys, homologs to the inner pair of CSD-forming Cys. Plants (P) have an additional Cys, C-terminal to the nested Cys motif. Selective loss of Cys residues has resulted in a single N-terminal Cys in some ERO1 genes (purple branches) such as in ERO1L in tapeworm (O) and Silk moth (K), and ERO1B in the Japanese ricefish. Key to branches: A, Mammals; B, Birds; C, Frog and turtle; D, Shark and Spotted gar; E, Fish; F, Fish; G, Shark, Frog and Coelacanth; H, Birds/Snake/Chameleon; I, Mammals; J, Sea squirt/Oyster/Sea urchin; K, Flies (Mosquito/Beetle/Bugs); L, Ants; M, Lancelet and Polychaete worm; N, Worms; O, Tapeworms; P, Plants. **(B)** Line diagram of ERO1 showing the disulfides and NTR. The two CSDs are the two nested disulfides in the NTR region. The NTR region comprising residues 1–55 is typically deleted in yeast functional assays. An equivalent region is typically deleted in ERO1α (Hansen et al., 2012). Interestingly, this region is not generally deleted in ERO1B (Pagani et al., 2000).

The phylogenetic tree for ERO1, built from 119 sequences is shown in **Figure 4A**. Originally present as a single gene, ERO1 duplicated prior to the divergence of bony fishes. The single gene in organisms predating bony fishes is generally termed ERO1-like (ERO1L) because it is clearly homologous to yeast ERO1 but has diverged in some crucial aspects, for example the yeast sequence has a long C-terminal tail. Yeast ERO1 has diverged sufficiently from human ERO1 that a meaningful sequence alignment over the full mask region employed for the phylogenetic tree is not possible. Of the duplicated daughters, ERO1A is ubiquitously expressed. This gene is located on chromosome 14 in humans. The other daughter, ERO1B, expressed in secretory tissues such as the pancreas, is found on chromosome 1 in humans. In metazoan ERO1L and ERO1A, the NTR has four Cys residues homologous to the CSD residues of human ERO1A, suggesting the CSDs are conserved across the group. In ERO1B, the four Cys are retained in the NTR of fishes and sharks, but only two Cys, homologous to those forming the inner CSD, are present in ERO1B of amphibians and higher teleosts. The presence of four Cys or two Cys in the NTR of ERO1B homologs partitions with sea and land animals respectively. Similar to ERO1B, in yeast ERO1, only the inner Cys pair is present.

What is the significance of Cys mutations in the NTR to the function of ERO1 homologs? As proteins rich in disulfides are highly expressed in secretory organs, the cells in these tissues are more susceptible to ER stress. Thus, ERO1B is likely to be under increased demand for disulfide formation in the secretory tissues where it is expressed compared to ERO1 homologs in other tissues. In addition, the emergence of animals onto land likely increased the importance of secretory organs in dealing with the effects of dehydration. The fact that one of the CSDs is lost in the NTR of ERO1B found in secretory tissues of land animals, suggests its loss is associated with increased activity of ERO1B. This suggests the NTR may have a role in inhibition of ERO1. The loss of the outer CSD of ERO1B in land animals may have resulted in decreased inhibition, allowing these organisms to cope with

the greater demands for disulfide bond formation in their secretory organs. The fact that NTR-deletants are typically used for functional assays of ERO1A and yeast ERO1 is consistent with this hypothesis.

AKT

Kinases of the AKT family are cytoplasmic proteins forming essential components in growth factor signaling pathways, activated downstream of the membrane-bound phosphoinositol-3 kinase (PI3K). The AKT genes are serine/threonine-protein kinases involved in regulating many processes such as metabolism, proliferation, cell survival and growth, and angiogenesis. AKT1 (RAC-α) is encoded on chromosome 14 of the human genome. AKT2 (RAC-β), which acts in the insulin signaling pathway, is encoded on chromosome 19. Both AKT1 and AKT2 are activated by platelet-derived growth factor. AKT3 is expressed in the brain.

The protein consists of three domains: an N-terminal Pleckstrin homology (PH) domain, a protein kinase domain, and a C-terminal AGC kinase domain. The PH domain is involved in membrane targeting of AKT via binding of phospholipids. The CSD is formed between Cys 60 and Cys 77 in the PH domain (PDB: 1unr). Crystallographic evidence supports redox activity of the disulfide (Fan et al., 2009). The reduced structure is PDB 1unq. It undergoes a morphing transition: a plastic deformation involving a large-scale conformational rearrangement of the polypeptide backbone which is likely regulated by redox activity of the CSD (Fan et al., 2009). Cys 77 can be modified by methylglyoxal to form an advanced glycation end product (AGE). Methylglyoxal is a byproduct of several metabolic pathways including threonine metabolism, lipid peroxidation and glycolysis.

Like CD4, AKT has multiple redox-regulated thiol sites. In AKT2 a redox-regulated order/disorder transition, modulated by reduction of the Cys 297/Cys 311 disulfide in the T loop, controls exposure of a key phosphorylation site at Thr 309 (Huang et al., 2003; Fan et al., 2009). Formation of the T loop disulfide is proposed to inhibit kinase activity by recruiting the cognate phosphatase (Leslie, 2006). Grx reduces AKT, although it is not clear whether it acts at the CSD, the T loop disulfide, or some other site (Murata et al., 2003). Modification of Cys 77 by methylglyoxal results in activation of AKT1, promoting proliferation of vascular smooth muscles (Chang et al., 2011). Overexpression of an AKT1 Cys77Ser mutant increased cell proliferation and DNA synthesis which could not be augmented by methylglyoxal treatment like the wild-type.

The phylogenetic tree for AKT built from 262 sequences is shown in **Figure 5**. The AKT gene has triplicated from the original ancestor: The original AKT-like (AKTL) gene is found in metazoa ranging from *C. elegans* to lamprey. An initial duplication, occurring after the divergence of lamprey, a jawless fish, was followed shortly after by a second duplication. AKTL genes predominantly have a single N-terminal Cys, for example all arthropods; but orthologs exist with no Cys (clade Q—eye worm, pig round worm and pinewood nematode worm; and the red flour beetle within clade W, Coleoptera); and a single C-terminal Cys (clade R-*C. elegans*, pole worm and nematode worm, clade O—Sea squirt). Clade N is the earliest metazoan with both Cys of the CSD. This group includes the hemichordate acorn worm and a placazoan, a basal form of invertebrate. Lamprey is the highest organism containing a single AKT gene with both Cys of the CSD. Subsequent to the divergence of lamprey the AKT gene underwent a triplication, with all daughters: AKT1, AKT2, and AKT3, retaining the CSD.

PHYSICOCHEMICAL PROPERTIES OF THE CSDs

The physicochemical properties of the CSDs of interest to this study are listed in **Table 1**, along with those of three control disulfides. Two positive controls are barley α-amylase/subtilisin inhibitor (BASI) Cys 144–Cys 148, a CSD which is a preferred substrate of Trxh; and DsbD Cys 103–Cys 109, a CSD which is the active site disulfide in the N-terminal domain of DsbD, which is reduced *in vivo* by the Trx-like C-terminal domain of DsbD. A negative control is BASI Cys 43–Cys 90 which is reduced by Trxh, but is not a preferred substrate (Maeda et al., 2006). All the CSDs adopt the right-handed staple conformation and have medium torsional energies (10–17.5 kJ.mol^{-1}) typical of CSDs, except the outer disulfide of the ERO1 nested pair. This disulfide between Cys 35 and Cys 48 adopts a much higher energy *cis* GGS′ conformation (Haworth et al., 2007). Its extremely high torsional energy is consistent with an auto-reduction process; the Cys of this disulfide could potentially isomerize with Cys 37 and Cys 46 or may even be mechanically reduced. The negative control in BASI between Cys 43 and Cys 90 is a low energy disulfide in a right-handed spiral conformation, typical of structural disulfides (Haworth et al., 2007). In summary, with the exception of the Cys 35–Cys 48 CSD of ERO1, all the CSDs in the proteins of interest had conformations and torsional energies consistent with CSDs which are preferred substrates of Trx-like enzymes.

Finally, we attempted to assess the likelihood that the reduced form of the protein of interest was a likely target of ROS/RNS; and which of the Cys was more likely to be modified. This was only possible for the PH domain of AKT. The controls we used were reduced forms of Trx-like enzymes and other proteins known to be modified by GSH. The results are shown in **Table 2**. The pKas of the active Cys in Grxs were generally between 7 and 8, while Cys in proteins which are modified by GSH were between 10 and 11. The values calculated for AKT were in the same range as GSH substrates. From the point of view of pKa, both Cys are equally likely to be attacked by ROS/RNS. However, other considerations may be important *in vivo* such as solvent accessibility of the individual Cys i.e., whether it is buried or accessible.

DISCUSSION

Disulfide bonds between Cys residues are generally thought to confer extra rigidity and stability to their resident protein, forming a type of proteinaceous spot weld. The conundrum of disulfides as structural stabilizers is how the two Cys residues could have been introduced into the protein chain simultaneously. A possible explanation is suggested by the emerging paradigm that the disulfide proteome consists of two subproteomes: a structural group and a redox-sensitive group (Yang et al., 2007). For structural disulfides, both Cys are functionally required. However, a single Cys residue can form a redox-sensitive site on a protein.

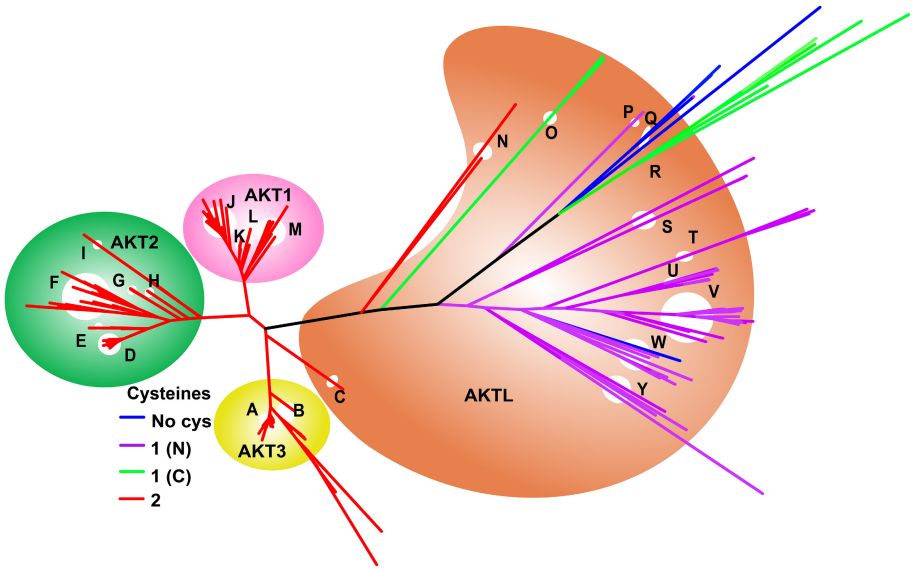

FIGURE 5 | Evolution of AKT genes showing the presence of the CSD. Colors of branches denote the number of Cys residues homologous to those of the CSD present in each species. Reversion to a single C-terminal Cys in the Chinese tree shrew is accompanied by rapid diversion of the sequence from other mammalian sequences. Keys to branches A, Mammals, birds, reptiles; B, Fishes; C, Sea lamprey; D, Mammals; E, Snakes; F, Spotted gar, salmon, and fishes; G, Frogs; H, Elephant fish; I, Zebra fish; J, Mammals; K, Tasmanian devil, Opposum, Turtle, and Birds; L, Frog and Coelacanth; M, Fishes; N, Acorn worm; O, Sea squirt; P, Trichina worm; Q, Eye worm, Pig round worm, and Pinewood nematode worm; R-*C. elegans*, pole worm and nematode worm; S-Y-Arthropods including Lepidoptera (T) and Diptera (U,V).

Table 1 | Dihedral angles calculated for the disulfides of interest in the proteins studied with the torsional energies and their respective conformations.

Protein	PDB code	Disulf	χ_1	χ_2	χ_3	χ'_2	χ'_1	Torsion energy kJ/mol	Conf.	Motif
AKT	1unr	60–77	−52.3	−120.5	105.2	−82.6	−50.9	13.9	Staple	CSD
ERO1	3ahq	35–48	−54.3	−121.9	45.8	75.6	176.7	36.9	*cis*	CSD
ERO1	3ahq	37–46	−61.3	−79.9	110.1	−102.8	−58.8	11.7	Staple	CSD
CD4	4h8w	130–159	−67.0	−86.3	108.2	−91.1	−58.6	11.7	Staple	CSD
+BASI	1ava	144–148	−63.2	−93.9	115.6	−78.1	−71.0	12.5	Staple	CSD
−BASI	1ava	43–90	−60.1	−73.5	−89.3	−73.6	−67.0	1.6	Spiral	–
+DsbD	1jpe	103–109	−51.0	−116.4	92.1	−77.8	−72.8	13.8	Staple	CSD

Thus, a redox-active disulfide may be introduced into a protein structure by stepwise mutation of two residues in the native sequence to Cys. By extension, evolutionary acquisition of structural disulfides in proteins can potentially occur via transition through a redox-active disulfide state.

Alternatively, a fully-fledged disulfide of either type could be acquired via a retrotransposon. Indeed, introduction of another type of redox site, the four Cys zinc (Zn^{2+}) binding site (Wouters et al., 2010), into proteins appears to largely have been effected by retrotransposons (Babu et al., 2006). A hallmark of this type of acquisition is insertion of a sequence fragment. In the case of Zn fingers, the fragment is around 30 amino acids in length.

The cases of CSD acquisition here clearly do not involve insertion of a sequence fragment, as evidenced by the sequence alignment. Instead, the redox switch was introduced by stepwise mutation of two residues in the native sequence to Cys. The advantages of acquisition of the CSD to protein function are clearly demonstrated by the retention of the redox switch in higher organisms. It is interesting to note that triplication of the AKT gene occurred after acquisition of the CSD, and it remained encoded in all daughter genes, suggesting it is integral to the function of these genes. In some species or homologs, the CSD acquisition process appears to have reversed. For example, in ERO1B, one of the CSDs was lost in secretory tissues of land animals.

Do the two Cys need to be acquired in a particular order during the evolutionary process? In order to be glutathionylated, a minimum requirement is that the Cys has a low pKa. When two disulfide-forming Cys are present, the Cys with the lower pKa is generally termed the "active" Cys (**Figure 1**), because it is the one targeted by ROS/RNS. The second Cys, which prevents

Table 2 | Calculated pKas for AKT and control proteins.

Protein	Cys	PDB	pKa
ScGrx6	136	3l4n	7.42
StGrx2	9*	3ir4	7.11
HsGrx2	37	2fls	8.01
HsCLIC1	24	1k0n	9.43
SpGST	10*	1f2e	11.56 ± 0.24
PmGST	10*	1pmt	11.07
BxGST	10	2dsa	17.29 ± 0.05
HsGSHR	58*	1dnc	9.52 ± 0.11
TttRNA methyltransferase	223	3g5s	10.09
Rncarbonic anhydrase	183*	1flj	10.57
AKT	60	1unq	11.35
AKT	77	1unq	11.03

**Indicates the proteins where GSH was removed from Cys for calculation. Standard error was calculated where required.*

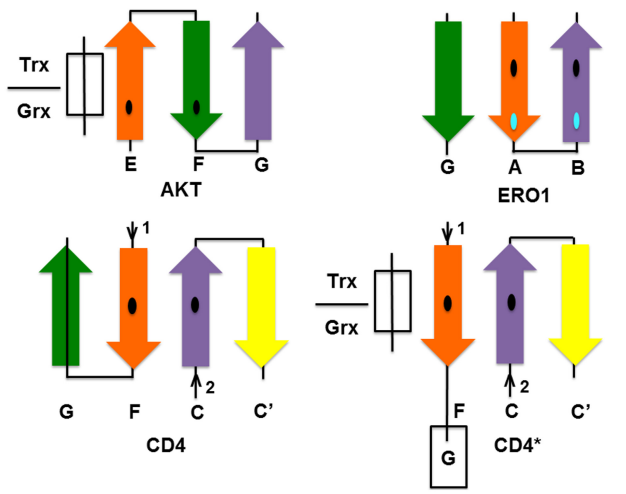

FIGURE 6 | Location of CSDs in the secondary structure of the proteins studied according to PDB structures: CD4—4h8w, ERO1—3ahq, and AKT—1unr. The Cys on the orange strand is the putative active Cys for each protein. Trx/Grx must bind as an extra strand near the active Cys as shown for AKT. In ERO1 and CD4, this site is protected by an additional strand (strand G in both CD4 and ERO1), which must be displaced to allow access by Trx. For CD4 to become accessible to Trx/Grx, one of the hairpins must hinge open to allow Trx/Grx to dock in place of the strand as shown in the hypothetical CD4* diagram.

overoxidation of the active Cys by disulfide formation, is termed the "resolving Cys." However, the pKas of the two Cys are not easily discerned from a protein structure and are generally determined experimentally. In addition, the structural requirements of Grx and Trx docking are such that the active Cys should be on an edge strand (**Figure 6**). Thus, by the simplistic hypothesis depicted in **Figure 1**, the low pKa Cys should be introduced into the protein first and be mutated from the protein last; and it should be on an edge strand. How does this hypothesis stack up with the three proteins studied here? The relevant information is summarized in **Table 3**.

Table 3 | Contexts of CSD residues in proteins of interest.

Protein	N-Cys	Edge?	C-Cys	Edge?	Environment
CD4	130	No	**159**	No	Cell surface
ERO1	**35**	No	48	Yes	ER
ERO1	**37**	No	46	Yes	ER
AKT	**60**	Yes	77	No	Cystosol

The table is based on PDB structures 1unr (AKT), 4h8w (CD4), and 3ahq (ERO1). The most common 1-Cys residue in the sequence alignments is bolded. This is likely to be the "active" Cys.

Of the three molecules, only AKT can dock Grx/Trx in the manner suggested, as shown in **Figure 6**. However, of the three molecules, AKT is the only cytosolic protein and thus would be found in the reduced state under normal physiologic conditions. Thus, consideration of the cellular compartment is an additional complication. Oxidation of a cytosolic molecule, promoting formation of the disulfide-bonded form, will only occur under conditions of oxidative stress. Disulfide bond formation in the pleckstrin-homology domain of AKT is likely associated with membrane targeting of AKT under conditions of oxidative stress (Fan et al., 2009). The likelihood that Cys 60 is the active Cys of the AKT CSD is supported by studies which demonstrate its modification in experiments on growth factor signaling (Antico Arciuch et al., 2009). On the other hand, CD4 and ERO1 exist in oxidative environments where the disulfide bond would be formed in the latent state: CD4 is cell surface protein of the blood plasma; ERO1 is an ER-resident protein.

Only in the cytosolic protein AKT, where the disulfide is normally in the reduced state, is the protein readily accessible by Trx. In CD4 and ERO1, the putative Trx-binding site is blocked by an additional strand. In CD4, the additional strand G is formed by residues 166–172 (PDB: 4h8w). In ERO1, strand G is formed by residues 355–359. Protection by an additional strand may be a general feature of CSDs in oxidative environments to prevent adventitious reduction of the CSD.

Because the disulfides in ERO1 and CD4 are latently oxidized, it is likely that Trx is only given access to the site under special conditions. For example, in CD4 the site becomes accessible during T-cell signaling. It has been proposed that CD4 undergoes domain swapping to facilitate this reduction (Matthias et al., 2002), a process that involves rearrangement of β strands. We speculate on the nature of this process in CD4 below. The latently oxidized states, are thus likely "occult" Trx-binding sites which must be unmasked by conformational changes prior to Trx binding and disulfide reduction (Wouters et al., 2010).

Several other proteins containing CSDs domain swap suggesting a relationship between domain swapping and CSD reduction (Wouters et al., 2010). GSH binding may also be related to domain swapping: GSH binding to Cys 60 of glyoxalase I regulates domain swapping in this protein (Saint-Jean et al., 1998).

EVOLUTION OF CD4

Several evolutionary studies of CD4 have examined the non-synonymous/synonymous substitution rate ($\omega = dN/dS$) in order to determine which regions of the CD4 molecule are under

positive selection. Non-synonymous changes are nucleotide codon changes that alter the amino acid coded at the protein level, while synonymous changes alter the nucleotide while preserving the amino acid. These studies found that CD4 is evolving rapidly under positive selection. In a pairwise comparison of human and mouse CD4, Ansari-Lari et al. (1998) determined $\omega = 0.77$, much higher than the average of $\omega = 0.12$ determined for ∼13000 pairs of human/mouse orthologs. A study of primate CD4 sequences identified positive selection ($\omega > 1$) throughout the molecule, but chiefly in the N-terminal region corresponding to D1 and D2 (Zhang et al., 2008). The sequence up to residue ∼153, which includes the first Cys residue of the CSD, has stronger selection than average for the molecule ($\omega > \omega^{-} = 1.17$).

Our study shows that the relatively rapid evolution of CD4 is predominantly focused on acquisition and modification of thiols involved in redox signaling. CD4 is an interesting molecule from the point of view of evolution of thiol-based redox signaling. All three known protein-protein interaction sites contain important cysteine residues. The primordial cysteine site: the CXC motif found in the cytoplasmic tail, is not found in the ancestral gene LAG3. This motif mediates the interaction with p56LCK necessary for T cell activation (Laing et al., 2006).

A second site is the CSD in D2, which is the subject of this study. This is the primary recognition site for HIV gp120 and a co-recognition site for MHCII molecules. The independent acquisition of the CSD in two clades, primates and muroids, at roughly the same epoch ∼75 MYA is interesting, and could arise from an environmental change, for example in the oxygenation of the Earth's atmosphere. This date is not long after the KT extinction, the global event that wiped out the dinosaurs around 80 MYA. Alternatively the dual acquisition might have a biological origin e.g., challenge by a common parasite or infectious agent. Mammals evolved during an interesting period of the earth's history with respect to continental drift. During the creataceous period, the super-continent Gondwana broke up. Given the co-location of the two clades involved on the same continent, the second "biological challenge" hypothesis seems attractive. The pairwise sequence alignments show the CSD site has been undergoing rapid evolution since its introduction in both clades (Figure S2).

The third protein-protein interaction site bearing important Cys residues is located in D1. Interestingly, in a process similar to step 1 of the CSD evolution in D2, modification of the canonical Ig disulfide between strands B and F has also occurred in D1 of some fish CD4 proteins (trout and fugu), which contain a distinct unpaired Cys in strand F, but lack the second Cys in strand B that forms a disulfide in mammalian and bird CD4 molecules.

Finally, LAG3 is very similar to CD4 but lacks the LCK binding site (Laing et al., 2006) (α in **Figure 2B**). Functionally LAG3 interacts with MHCII with higher affinities than CD4, and may have a role in impeding access to MHCII of CD4 (Workman et al., 2002; Workman and Vignali, 2003). The two Ig-domain molecule CD4REL (β in **Figure 2B**) appears to be a progenitor of CD4 which is also found in the lamprey a vertebrate which diverged earlier than fish. The lamprey genome does not contain a CD4 ortholog (***γ*** in **Figure 2B**). Trout contains both CD4REL and CD4, but vertebrates that evolved later, such as the chicken, do not appear to have a CD4REL-like molecule, retaining only CD4.

Thus, CD4 arose *de novo* in fish around 450 MYA around the time its primary tissue of origin, the thymus, appeared in animals. Originally containing the important cytosolic CXC motif, CD4 then underwent rapid evolution at two distinct protein-protein interaction sites in D1 and D2, both bearing important Cys residues.

Speculations on activation of CD4 by HIV gp120

Given the importance of CD4 CSD reduction in the entry of HIV gp120 into the cell, it is worth speculating on the nature of this process in the light of the hypothesis put forward here. Sheet 1 in D2 of CD4, which consists of residues 99–119 and 142–146, can be considered as the "torso" of D2 from which two β-hairpin "arms" are extended, comprising residues 127–140 (N-arm) and residues 157–172 (C-arm). These "arms" are clasped together by a series of hydrogen bonds and the disulfide formed between Cys 130 and Cys 159 on strands C and F to form the second sheet. In domain swapping, either one or both of these arms may be exchanged with an adjacent CD4 molecule. This can only be achieved by breakage of the C-F hydrogen bonds and reduction of the CSD between Cys 130 and Cys 159. In its location on the interior strands of the four-stranded sheet 2, the CSD is protected from interaction with Trx and Grx. However, it may be accessible by GSH. In order to become accessible to Trx/Grx, one of the hairpins must hinge open, to allow Trx/Grx to dock in place of the strand. As Cys 159 is conserved in all CD4 molecules, it is likely the "active Cys." Thus, docking of Trx/Grx may be effected by peeling away the C-terminal strand (G) from the molecule (CD4* in **Figure 6**). Thus, we propose this is a necessary "priming" step for CD4 CSD reduction.

CONTEXT-DEPENDENCE OF Cys INCORPORATION IN PROTEIN SEQUENCES

Early studies on protein sequences indicated Cys is more abundant in more complex organisms (Fahey et al., 1977; Miseta and Csutora, 2000). This has been confirmed by more recent studies on whole genomes that have shown the abundance of Cys in proteins has been increasing since divergence from the Last Universal Common Ancestor (Woese, 1998; Brooks and Fresco, 2002); and this increase is a continuing panspecies phenomenon which is evident within the last 10 million years (Jordan et al., 2005). It seems likely that increased Cys abundance arises from increased use of thiol-based redox signaling in more complex organisms. Although the prevalence of Cys in proteins is increasing, the abundance has not yet reached the neutral frequency (i.e., the frequency that would be expected based on the number of Cys codons in the genetic code) (King and Jukes, 1969; Brooks and Fresco, 2002). The failure of Cys to reach its equilibrium frequency suggests that incorporation of additional Cys into proteins may come at a cost: increased signaling and control may be gained at the cost of deleterious effects of over-oxidation (Wouters et al., 2010).

This study suggests that successful incorporation of Cys may be limited to very select contexts in proteins, specifically those where reduction of the Cys can be effected by enzymes of redox

homeostasis. In this study, the successfully incorporated sites are likely reduced by Trx-like enzymes. The disulfide bond in CD4 is reduced by Trx (Anikszteijn et al., 1987; Matthias et al., 2002), and the disulfide in AKT may be sensitive to Grx (Murata et al., 2003; Wang et al., 2007).

If a Cys that is modifiable by GSH is in a place which cannot be accessed by Grx, the protein will likely be targeted for removal by the GSH detoxification system. Thus, the mutation most likely will have a deleterious effect on protein function by inducing a state of haploinsufficiency. If the Cys is in a place where it may be reduced by Grx, introduction of the Cys does not have a deleterious effect on protein function because it may be removed. If, in addition, it is introduced in a place which modulates the protein's function in a way which is beneficial to redox function, it may become fixed in the population. For example, if addition of GSH temporarily prevents a protein interaction that would be deleterious under conditions of oxidative stress. Thus, only 1-Cys GSH switches that are beneficial to physiologic function are likely to be retained in the population sufficiently long for random mutation of a second Cys to occur; and this second mutation must also be sufficiently beneficial to be retained over the 1-Cys variant.

CONVERGENT ACQUISITION OF MODULAR REDOX SWITCHES

Before multiple genome sequences were complete, it was generally believed increased complexity of organisms correlated with gene number. After completion of the first genomes, the small differences in gene number between simple unicellular eukaryotes and mammals forced revision of how complexity is encoded. For example, the yeast genome contains 5000 genes, yet the human genome contains only five times more: 25,000. Additional complexity at the organismal level is likely encoded at the molecular level by noncoding DNA, as this is considerably different between these organisms (Mattick, 2004).

However, increased complexity may also be encoded at the protein level. It was previously recognized that concatenation of existing domains through gene fusion, also known as protein domain mosaicism, encodes new functions in more complex organisms (Patthy, 1985). Studies on the changing amino acid content of proteins show that domains also are not static structures. Additional complexity added to protein domains in the form of redox and other switches likely increases the signaling capabilities of individual domains. In other words, nature is continually tinkering with these independent folding units: a domain from archaea may not have the same sophisticated set of switches as the homologous domain from a mammalian protein.

Thus, two modes of acquisition of increased protein complexity have been demonstrated to date: protein domain mosaicism (Patthy, 1985), and acquisition of allosteric control sites. Previously, Babu et al. (2006) showed that Zn finger allosteric control sites are added to protein sequences via retrotransposons. Here we studied a novel method of acquisition of an allosteric control site: formation of CSDs by stepwise acquisition of Cys residues in appropriate contexts. Both these Cys-based sites, Zn fingers and CSDs, are known to be redox regulated.

In this study, we analyzed incorporation of disulfide-based redox switches into three protein families. Successful context-dependent stepwise evolution of the sites likely enabled the proteins to utilize existing Trx-like enzymes to mediate successful incorporation into cellular mechanisms of redox homeostasis. Thus, three non-homologous proteins have convergently acquired the same modular redox switch: the CSD. CSDs are, by far, the most common forbidden disulfide found in homologous sites of protein structures (Wouters et al., 2010). They have evolved independently multiple times. CSDs have been postulated to have special redox properties: specifically intermediate torsional energies that enable them to be reduced in a controlled process by other thiol sites in proteins. However, many other forbidden disulfides share this property, so this does not explain why evolution of CSDs is favored over other forbidden disulfides. Work by Maeda et al. (2005), which suggests that a CSD in BASI is specifically recognized by Trx; and our own bioinformatic work, which has identified multiple CSDs which undergo redox reactions with the CXXC motif of Trx-like enzymes, suggest that CSDs are cognate substrates of Trx-like enzymes (Haworth and Wouters, 2013). Although Trxs recognize multiple non-homologous substrates, solved protein structures of disulfide-linked Trx-substrate reaction intermediate mimics have provided evidence for recognition of structural motifs in target proteins by Trxs (Qin et al., 1995, 1996; Maeda et al., 2006; Chartron et al., 2007).

Although BASI has nine disulfides, only two are specifically targeted by Trxh (Maeda et al., 2005). Cys 144–Cys 148 and Cys 43–Cys 90. Of these two, Cys 144–Cys 148, which is a CSD, is much more efficiently reduced. The specificity of the reaction is partly ensured by consistent docking of the substrate. In most solved protein structure complexes, Trx-like molecules bind their substrates as an antiparallel β-strand. The interaction between Trx and its substrate is characterized by three distinctive hydrogen bonds of a pattern typically found between pairs of antiparallel β-strands. A similar pattern of hydrogen bonds is observed between the N-terminal Trx-like domain of *Escherichia coli* DsbD in complex with the C-terminal domain of DsbD which contains the CSD (Cys 103–Cys 109) substrate (Rozhkova et al., 2004). This suggests the evolution of CSDs is favored over other forbidden disulfides because they can be immediately exapted from the Grx to the Trx redox homeostasis pathways.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphar.2015.00001/abstract

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The still mysterious roles of cysteine-containing glutathione transferases in plants

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Glutathione transferases (GSTs) represent a widespread multigenic enzyme family able to modify a broad range of molecules. These notably include secondary metabolites and exogenous substrates often referred to as xenobiotics, usually for their detoxification, subsequent transport or export. To achieve this, these enzymes can bind non-substrate ligands (ligandin function) and/or catalyze the conjugation of glutathione onto the targeted molecules, the latter activity being exhibited by GSTs having a serine or a tyrosine as catalytic residues. Besides, other GST members possess a catalytic cysteine residue, a substitution that radically changes enzyme properties. Instead of promoting GSH-conjugation reactions, cysteine-containing GSTs (Cys-GSTs) are able to perform deglutathionylation reactions similarly to glutaredoxins but the targets are usually different since glutaredoxin substrates are mostly oxidized proteins and Cys-GST substrates are metabolites. The Cys-GSTs are found in most organisms and form several classes. While Beta and Omega GSTs and chloride intracellular channel proteins (CLICs) are not found in plants, these organisms possess microsomal ProstaGlandin E-Synthase type 2, glutathionyl hydroquinone reductases, Lambda, Iota and Hemerythrin GSTs and dehydroascorbate reductases (DHARs); the four last classes being restricted to the green lineage. In plants, whereas the role of DHARs is clearly associated to the reduction of dehydroascorbate to ascorbate, the physiological roles of other Cys-GSTs remain largely unknown. In this context, a genomic and phylogenetic analysis of Cys-GSTs in photosynthetic organisms provides an updated classification that is discussed in the light of the recent literature about the functional and structural properties of Cys-GSTs. Considering the antioxidant potencies of phenolic compounds and more generally of secondary metabolites, the connection of GSTs with secondary metabolism may be interesting from a pharmacological perspective.

Keywords: cysteines, deglutathionylation, glutathione transferases, photosynthetic organisms, phylogeny

INTRODUCTION

Glutathione is a tripeptide with the sequence γGlu-Cys-Gly that is mostly present in reduced (GSH) or disulfide (GSSG) forms, even though nitrosoglutathione (GSNO) may represent another important source. Under physiological conditions, free glutathione is present in concentrations ranging from 1 to 10 mM with the reduced form largely predominating over the oxidized form (Gutscher et al., 2008; Pallardo et al., 2009). As such, glutathione is the major non-protein thiol source in eukaryote cells, likely constituting a crucial redox buffer (Rouhier et al., 2008). Glutathione can also fulfill additional roles. In eukaryotes, glutathione is essential for a proper development, controlling in particular cell-cycle progression. Apart from development, glutathione is crucial for stress response by (i) neutralizing radicals, (ii) participating in heavy metal tolerance, either directly or as a constitutive element of phytochelatins, (iii) contributing to the regeneration of antioxidant molecules such as ascorbate and α-tocopherol, and (iv) providing electrons and protons to glutathione transferases (GSTs) or to peroxiredoxins, both with and without the involvement of glutaredoxins (Grxs) for peroxide removal (Rouhier et al., 2008). While the exact role of glutathione has not been completely defined, it is clear that, depending on its redox state, glutathione can react with various intracellular molecules and that glutathionylation/deglutathionylation reactions of both proteins and smaller compounds are central to GSH functions. Protein glutathionylation is a reversible post-translational modification that is now recognized as a major signaling or protective mechanism. It occurs under basal non-stress conditions but has mostly been documented in response to oxidative stress conditions (Zaffagnini et al., 2012). The reversible reaction i.e., deglutathionylation, occurs either by the intervention of Grxs or by direct thiol/disulfide exchange reactions with GSH once an appropriate GSH/GSSG ratio has been restored.

In addition to proteins, glutathionylation of metabolites has also attracted a lot of attention as it constitutes an intermediate step in a number of metabolic processes and detoxification pathways. It has been well-established that most organisms possess a three-step detoxification system to eliminate endogenous and exogenous toxic compounds (Coleman et al., 1997; Morel et al., 2013). In the first step, enzymes such as cytochrome P450 monooxygenases catalyze various reactions (oxidation, reduction or hydrolysis) to expose or introduce a functional moiety on hydrophobic substrates. In the second step, conjugating enzymes perform addition reactions (e.g., acetyl, methyl, glucuronic acid) on these newly modified, electrophilic substrates. The glutathione addition onto electrophilic molecules is well-recognized and is mediated by specific classes of GSTs having usually a serine or a tyrosine as a catalytic residue. Finally, glutathionylated products are either exported from the cells or sequestered in vacuoles. In plants, GSTs have been identified by showing glutathionylation of the herbicide atrazine (Lamoureux et al., 1970). Most subsequent studies have focused on these GST types that are here referred to as glutathionylating GSTs, although other biochemical activities have been described for some GST classes. To cite a few, numerous GSTs exhibit GSH-dependent peroxidase activities reducing simple peroxides but also organic hydroperoxides (Tang and Tu, 1994; Marrs, 1996; Hurst et al., 1998). Theta GSTs were shown to catalyze the isomerization of maleylacetoacetate into fumarylacetoacetate, a key component of the catabolism of tyrosine and phenylalanine (Thom et al., 2001; Fernandez-Canon et al., 2002). Besides these catalytic functions, GSTs could also exhibit ligandin functions, binding hydrophobic substrates in a so-called L-site for transport and storage purposes. In plants, it has been documented that GSTs with ligandin properties are not only implicated in the transport of anthocyanins and flavonoids but also of hormones such as auxin and cytokinin, which suggests a possible role in cell signaling (Smith et al., 2003; Kitamura et al., 2004; Moons, 2005).

With the increasing number of biochemical studies, it became clear that several GSTs do not have a glutathionylation activity but instead catalyze the opposite reaction; deglutathionylation (Dixon and Edwards, 2010a,b; Xun et al., 2010; Board, 2011; Meux et al., 2011). This capacity usually originates from the replacement of the catalytic serine or tyrosine residues in the active site motif by a cysteinyl residue as demonstrated for mammalian, insect and fungal Omega GSTs (GSTOs) (Board et al., 2000; Kim et al., 2006; Yamamoto et al., 2009; Meux et al., 2013), for plant Lambda GSTs (GSTLs) (Dixon and Edwards, 2010b; Lallement et al., 2014) and for bacterial and fungal glutathionyl hydroquinone reductases (GHRs) (Xun et al., 2010; Meux et al., 2011). However, the physiological functions of these enzymes have rarely been elucidated. Human GSTOs may be involved in arsenic biotransformation, reducing methyl and dimethyl arsenate (Zakharyan et al., 2001; Burmeister et al., 2008), whereas plant GSTLs may be involved in flavonoid metabolism and/or trafficking (Dixon and Edwards, 2010b). Interestingly, while the role of GSTs has classically been associated to the modification of small molecules and the role of glutaredoxins to the deglutathionylation of proteins, it has recently been shown that human GSTO1-1 can deglutathionylate β-actin, which should prompt us to consider proteins as GST substrates (Menon and Board, 2013). Concerning GHRs, the bacterial and fungal members characterized so far are involved in the catabolism of chlorinated quinones and in lignin degradation through the deglutathionylation of glutathionylated intermediates (Reddy and Gold, 2001; Masai et al., 2003; Huang et al., 2008; Meux et al., 2011). While these glutathionylated compounds constitute intermediates in catabolism pathways, they may also constitute intermediates for biosynthetic pathways as shown for sulfur-containing defense molecules such as camalexins or glucosinolates (Su et al., 2011). However, the roles of GSTs in the secondary metabolism are less documented compared to the ones of cytochrome P450 monooxygenases and to their involvement in xenobiotic detoxification. A plausible explanation is that intracellular GSH-conjugated compounds have rarely been successfully identified from plant extracts, possibly due to their transient nature or to the difficulty to isolate them. As examples, glutathionylated compounds have been identified as precursors of aromas in fruits (Fedrizzi et al., 2012; Peña-Gallego et al., 2012) or as conjugated oxylipins upon leaf infiltration of keto-fatty acids (Davoine et al., 2005).

Based on the biochemical properties of GSTs and their functional association with cytochrome P450 monooxygenases, acquiring fundamental knowledge about GST functions, regulation and substrates may be beneficial for diverse pharmaceutical and biotechnological applications. In biotechnology, the ability of some GSTs to catalyze GSH-conjugation reactions has been exploited not only for the development of sensitive biosensors or enzyme assays for the determination of the concentration of various pesticides and herbicides (Chronopoulou and Labrou, 2009) but also for the development of herbicide and stress-tolerant plants. Moreover, among the thousands of natural plant products, including polyphenols, flavonoids, alkaloids, and quinones, several molecules possess antimicrobial, anticarcinogenic, anti-inflammatory, or antioxidant properties (Lewis and Ausubel, 2006; Saleem et al., 2010) not to speak about unidentified or untested molecules. Although they are often relatively low abundant molecules, they also constitute a recognized source of molecules important for the cosmetic industry (fragrance) or for nutrition (gustatory perception/dietary complements) contributing to extend the color or aroma palette. For instance, GSTs, notably those from the Pi and Alpha classes, are known to be present in the olfactory epithelium and particularly in the covering mucus layer, where they would serve for metabolizing odorant molecules (Aceto et al., 1993; Debat et al., 2007). Overall, by recognizing and eventually modifying a wide range of antioxidant molecules, GSTs could represent promising enzymes in diagnosis and monitoring cancer invasion, liver, kidney, Alzheimer's and Parkinson's diseases (Chronopoulou and Labrou, 2009). They also have a considerable interest for isolating new secondary metabolites or for developing molecules (drugs or antimicrobial compounds) with different or improved pharmacological properties. As an example, Canfosfamide (TLK286, TELCYTA®), a cancer cell-activated prodrug, was designed to exploit the elevated levels and the activity of glutathione S-transferase P1-1 (GSTP1-1) that is overexpressed in many human cancer cells (Tew, 2005). Hence, GSTs could be useful for product transformation but also

for synthetic biology or metabolic engineering approaches, with the aim of generating new chemical entities.

Over the past years, the GST classification has constantly evolved, notably due to the increase of genomic data and to the presence of particular isoforms in a specific subset of organisms. The objective of this review is to present an overview of cysteine-containing GST (Cys-GST) classes in photosynthetic organisms by describing known data concerning the gene expression, the protein subcellular localization and their biochemical and structural properties.

THE GST FAMILY IN PHOTOSYNTHETIC ORGANISMS

The present phylogenetic analysis focuses on photosynthetic organisms and as a basis uses the Cd00570 Sequence Cluster of the "conserved domains" tool in NCBI, which includes the GST classes that contain the typical N-terminal thioredoxin (Trx) domain found in GSTs. This cluster is part of the thioredoxin superfamily, among other well-known clusters such as thioredoxin, glutaredoxin, peroxiredoxin, protein disulfide isomerase (PDI), and disulfide bond A (DsbA) oxidoreductase to name a few. The main criterion used is *a minima* the presence of the two classical GST domains, the N-terminal thioredoxin-like domain with a β1α1β2α2β3β4α3 topology and a C-terminal all-helical domain, that together form a typical GST fold. For this reason, Kappa GSTs and mPGES-1 (microsomal ProstaGlandin E-Synthase type 1), one subclass of MAPEGs (Membrane Associated Proteins in Eicosanoid and Glutathione metabolism) (Bresell et al., 2005), enzymes often integrated into the GST superfamily, are not considered here, even though some terrestrial plants and algae possess at least one mPGES-1 representative. The phylogenetic analysis of all GSTs found in eukaryote photosynthetic organisms has been fitted to the aforementioned criterion, which allows identification of 14 classes (**Figure 1**). The sequences used were those present in model organisms including a gymnosperm: *Pinus tabulaeformis*, several angiosperms: *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Solanum lycopersicum*, and *Hordeum vulgare*, a lycophyte: *Selaginella moellendorffii* and a moss: *Physcomitrella patens*.

Among these 14 classes, Tau, Phi, Zeta, Theta, and tetrachlorohydroquinone dehalogenase (TCHQD) classes clearly contain GSTs with a catalytic serine. The nature of the catalytic residue in the EF1Bγ and Ure2p classes is less clear, but RIRT and APNG motifs are found at a position similar to the active site signature in other GSTs. Finally, the seven other classes (Iota GSTs (GSTIs), Hemerythrin GSTs (GSTHs), Dehydroascorbate (DHA) reductases (DHARs), GSTLs, GHRs, mPGES-2s, and metaxins) contain members that clearly display a very conserved cysteine in the active site motif, hence suggesting that they belong to Cys-GSTs. Metaxins are part of the mitochondrial translocation system of the mitochondrial outer membrane, being anchored through their C-terminal region whereas the rest of the protein is oriented to the cytosol (Lister et al., 2007). However, they have not been integrated in this study. Indeed, although having the typical GST fold, none of the cysteine of the CPxC signature found in plant sequences is conserved in other organisms notably mammals, and there is no evidence for a cysteine involvement or for a requirement of GSH for their function.

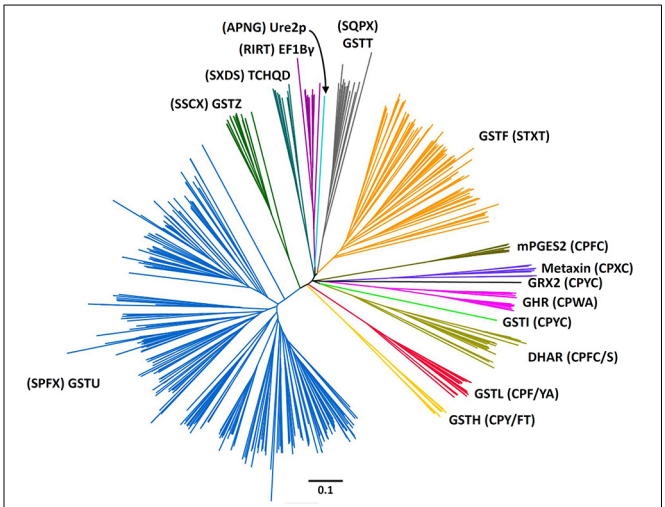

FIGURE 1 | Rooted phylogenetic tree of plant GSTs. The sequences used are those identified in *Arabidopsis thaliana* (Lan et al., 2009), *Hordeum vulgare* (Rezaei et al., 2013), *Oryza sativa* (Lan et al., 2009), *Physcomitrella patens* (Liu et al., 2013), *Pinus tabulaeformis* (Lan et al., 2013), *Populus trichocarpa* (Lan et al., 2009), and *Solanum lycopersicum* (Csiszar et al., 2014). Sequences were aligned with PROMALS3D and alignment manually adjusted with Seaview software (Gouy et al., 2010). The phylogenetic tree was constructed with BioNJ (Gascuel, 1997) in Seaview, rooted with *E. coli* glutaredoxin 2 and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of the branches was assessed by the bootstrap method with 500 replications. Various classes can be distinguished: Dehydroascorbate reductase (DHAR), Elongation factor 1Bγ (EF1Bγ), Glutathionyl hydroquinone reductase (GHR), Phi (GSTF), Hemerythrin (GSTH), Iota (GSTI), Lambda (GSTL), Theta (GSTT), Tau (GSTU), Zeta (GSTZ), Microsomal prostaglandin E synthase type 2 (mPGES-2), Tetrachloro-hydroquinone dehalogenase (TCHQD), and Ure2p. The scale marker represents 0.1 substitutions per residue. For clarity, the names of the sequences have not been indicated but all sequences are available in the Supplementary Material.

Concerning mPGES-2s, they were initially not considered as GSTs because GSH was not absolutely required for the detected activity e.g., the isomerization of ProstaGlandin H_2 (PGH_2) (Tanikawa et al., 2002) and because they exhibited a low similarity with GSTs identified at that time. However, based on (i) its typical GST structure, (ii) the identification of another activity strictly requiring GSH, and (iii) the identification of additional more closely related Cys-GSTs (Yamada et al., 2005; Takusagawa, 2013), mPGES-2s do in fact belong to the GST family. It is worth mentioning that Beta GSTs (GSTBs) and GSTOs as well as chloride intracellular channel proteins (CLICs), which belong to Cys-GSTs, are not found in plants. Moreover, particular proteins that are listed as putative GST members under the name "2-GST_N" have not been considered here. Although they have two repeated N-terminal Trx domains and a quite conserved CPFC motif in one of them, they lack the C-terminal domain. Since the GSTI and GSTH isoforms have not yet been characterized, we have essentially focused the following parts on the four remaining Cys-GST classes, describing in detail the current knowledge on DHAR, GHR, GSTL and mPGES-2 enzymes.

DISTRIBUTION OF CYSTEINYL GSTs IN PHOTOSYNTHETIC ORGANISMS: GENE CONTENT, STRUCTURE, AND ORGANIZATION

After retrieving all Cys-GST sequences present in representative cyanobacterial and algal genomes as well as in all completely sequenced terrestrial plant genomes, a comparative genomic analysis was performed to get an accurate classification of Cys-GSTs in the green lineage. The resulting phylogenetic tree confirmed six major clades corresponding to the 6 earlier defined classes (**Figure 2**). In previous phylogenetic analyses conducted with other gene families of the thioredoxin superfamily, e.g., thioredoxins, glutaredoxins, peroxiredoxins and PDIs, the gene structure (number of exons in eukaryotic genes) was conserved and coherent with the classes (Meyer et al., 2002; Rouhier and Jacquot, 2005; Rouhier et al., 2006; Selles et al., 2011). Here, the gene structure was not informative as it was not at all conserved among species, even the phylogenetically close ones.

The DHAR class is essentially present in terrestrial plants. Indeed, this class is absent in cyanobacteria and a single gene, that likely represents the ancestor DHAR gene, is found in a few algae of the chlorophyceae and trebouxiophyceae classes but not in prasinophyceae. The number of DHAR genes in a given species usually lies between 2 and 3 (**Table 1**). For species possessing a higher number of genes, the increase is not due to tandem duplication as the genes are neither found at adjacent positions nor on the same chromosome in most cases. In our chosen well-annotated reference genomes, there are two genes in *O. sativa* and *S. moellendorffii* and three in *A. thaliana*, *P. trichocarpa*, and *P. patens*. DHARs are split in two well-differentiated subgroups, the genes coding for chloroplastic proteins (CPFC active site motif) and those coding for proteins that are likely all cytosolic (CPFS active site motif) (**Figure 2**) with algal sequences being dispersed but on the same branch. Since algal genes code for proteins that are devoid of targeting sequences, the ancestral gene might be the one coding for the cytosolic members. Among terrestrial plants, all organisms have at least one member in each clade.

GSTLs appear unique to terrestrial plants, the number of genes ranging generally from 2 to 4 with the exception of *S. moellendorffii*, where the gene seems absent (**Table 1**). In *A. thaliana*, two genes (*AtGSTL1* and *AtGSTL2*) are repeated in tandem on the chromosome 5, likely indicating a recent duplication event. On the other hand, specific expansions have arisen in some species such as *Aquilegia coerulea*, *Malus domestica*, *Eucalyptus grandis*, and *Panicum virgatum* which have 5–8 genes. In this case, some events of tandem duplication have largely contributed to this increase. This is particularly true in *Eucalyptus grandis*, a species in which there are two gene clusters, one having a series of four

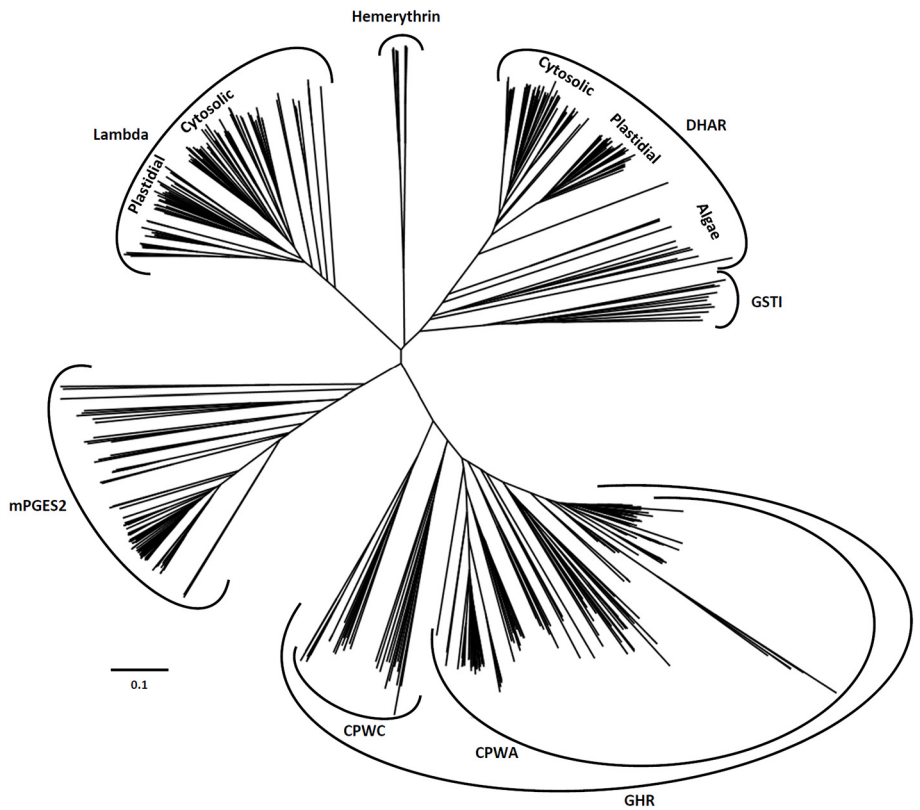

FIGURE 2 | Unrooted phylogenetic tree of Cys-GSTs present in the green lineage. Sequences were aligned with PROMALS3D using 1Z9H, 3PPU, and 4PQH PDB structures as templates. Then the alignment has been manually adjusted with Seaview software. The phylogenetic tree was constructed with BioNJ and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of the branches was assessed by the bootstrap method with 500 replications. The scale marker represents 0.1 substitutions per residue. For clarity, the names of the sequences have not been indicated but all sequences are available in the Supplementary Material.

Table 1 | Cys-GST gene content in sequenced chlorophytes and embryophytes.

	DHAR	GHR	GSTL	mPGES-2	GSTI	GSTH	Total
VIRIDIPLANTAE							
Chlorophyte							
Chlamydomonas reinhardtii v5.5	1	3	0	1	1	0	6
Chlorella sp. NC64A	1	2	0	1	1	0	5
Coccomyxa subellipsoidea C-169 v2.0	2	2	0	1	1	0	6
Micromonas pusilla CCMP1545 v3.0	0	2	0	1	1	0	4
Micromonas pusilla RCC299 v3.0	0	2	0	1	1	0	4
Ostreococcus lucimarinus v2.0	0	1	0	1	1	0	3
Volvox carteri v2.0	1	0	0	1	1	0	3
Embryophyte							
Physcomitrella patens v3.0	3	2	1	2	1	8	17
Tracheophyte							
Selaginella moellendorffii v1.0	2	5	0	1	1	2	11
Angiosperm							
Grass							
Brachypodium distachyon v1.2	2	2	2	1	0	0	7
Oryza sativa v7.0	2	2	3	1	0	0	8
Panicum virgatum v1.1	3	3	6	1	0	0	13
Setaria italica v2.1	2	2	4	1	0	0	9
Sorghum bicolor v2.1	3	2	4	1	0	0	10
Zea mays 6a	4	7	4	1	0	0	16
Eudicot							
Aquilegia coerulea v1.1	2	2	6	1	0	0	11
Pentapetalae							
Mimulus guttatus v2.0	2	2	3	2	0	0	9
Solanum lycopersicum iTAG2.3	2	2	5	1	0	0	10
Solanum tuberosum v3.4	2	2	3	2	0	0	9
Vitis vinifera Genoscope.12X	2	1	4	2	0	0	9
Rosid							
Poplar-Malvidae							
Eucalyptus grandis v1.1	3	3	8	3	0	0	17
Populus trichocarpa v3.0	3	2	3	3	0	0	11
Brassicales-Malvales							
Carica papaya ASGPBv0.4	2	0	2	1	0	0	5
Gossypium raimondii v2.1	3	2	3	2	0	0	10
Theobroma cacao v1.1	2	3	2	2	0	0	9
Brassicaceae							
Arabidopsis lyrata v1.0	3	4	2	1	0	0	10
Arabidopsis thaliana TAIR10	3	4	3	1	0	0	11
Boechera stricta v1.2	3	4	3	1	0	0	11
Brassica rapa FPsc v1.3	5	4	3	1	0	0	13
Capsella grandiflora v1.1	3	4	2	1	0	0	10
Capsella rubella v1.0	3	2	1	1	0	0	7
Eutrema salsugineum v1.0	3	3	3	1	0	0	10
Citrus							
Citrus sinensis v1.1	2	2	3	2	0	0	9
Citrus clementina v1.0	2	2	3	2	0	0	9
Fabidae							
Linum usitatissimum v1.0	5	4	4	2	0	0	15
Manihot esculenta v4.1	2	1	2	2	0	0	7
Ricinus communis v0.1	3	2	3	1	0	0	9
Nitrogen-fixing							
Cucumis sativus v1.0	2	2	3	2	0	0	9
Fragaria vesca v1.1	2	2	3	2	0	0	9
Glycine max Wm82.a2.v1	4	2	5	3	0	0	14
Malus domestica v1.0	8	5	6	4	0	0	23
Medicago truncatula Mt4.0v1	2	1	4	2	0	0	9
Phaseolus vulgaris v1.0	2	2	4	2	0	0	10
Prunus persica v1.0	2	2	2	2	0	0	8

Sequences have been retrieved from Phytozome 10 and Joint Genome Institute databases. They are provided as Supplementary Material.

genes in a row. In the phylogenetic tree, the genes coding for the chloroplastic and cytosolic isoforms clearly separate into two groups. Since the single *GSTL* gene found in *P. patens* encodes a chloroplastic protein, the ancestral version in the green lineage should be the chloroplastic-encoding gene. On the other hand, the absence of *GSTL* genes in cyanobacteria and algae raises the question of the appearance of these isoforms. In a few cyanobacteria and algae, there are orphan, non-annotated sequences sharing similar active site motifs (CPYA). For this reason, it is tempting to speculate that these sequences might correspond to the ancestral gene and that it has been lost in most organisms. The fact that the overall similarity of these orphan sequences with GSTLs is low and that they do not necessarily form a single clade with GSTLs could come from their rapid and independent evolution. This will have to be further explored when additional genomes and sequences will be available.

The GHR class is widespread, with at least one gene present in almost all analyzed photosynthetic organisms and in most species. There are between 2 and 4 GHR genes (**Table 1**). The absence of gene in some species might be due to either annotation problems or gene loss. Furthermore, the gene family expansion found in some species, e.g. *Zea mays*, *S. moellendorffii*, and *M. domestica* cannot be explained by tandem duplication in this case. For *M. domestica*, which has by far the highest number of Cys-GST genes (23 genes) and exhibits gene expansion in all classes, this can be explained by a recent genome-wide duplication (Velasco et al., 2010). The widespread nature of GHRs is also true outside photosynthetic organisms since they are present almost everywhere including archaea of the halobacteriaceae order, but excluding mammals (**Table 2**). Overall, this suggests that GHRs have crucial functions, or at least functions that cannot be ensured by other GSTs.

Regarding the mPGES-2 class, its genes are absent in cyanobacteria whereas at least one gene is present in algae and terrestrial plants. This suggests that mPGES-2 proteins may have a widespread and essential function. Since most organisms retained only one gene, the duplication observed in some specific organisms probably derives from isolated events. Additionally, these GSTs are also largely distributed among kingdoms since they are found in mammals, nematodes, insects, and trypanosomatids but not fungi (**Table 2**).

The last two classes, GSTI and GSTH, are restricted to specific organisms. The GSTIs are found as a single gene in some cyanobacteria, algae, and in non-vascular plants (*S. moellendorffii*, and *P. patens*). In the phylogenetic tree, they form a single clade that is close to DHAR, possibly indicating that DHARs derive from GSTIs. The fact that GSTIs have been lost at some steps in the green lineage evolution and are no longer present in most terrestrial plants may also indicate that the associated function(s) disappeared or that other GSTs fulfill similar roles. The distribution of GSTHs is even more puzzling as from current available genomes, they are only found in *S. moellendorffii* and *P. patens*. The presence of 8 genes in *P. patens* is particularly striking, taking into account that, from the analysis of EST sequences, there is evidence for the expression of six genes. In *P. patens*, all these genes form a single gene cluster that likely originates from several duplication events.

Table 2 | Characteristics and distribution of Cys-GSTs.

Class	Origin	Typical catalytic motif	Average amino acid length	Oligomerization state
GSTB	Bacteria	$GA_{12}CS$	210	Dimer
GSTO	Mammals, insects, fungi	$_{35}$CPFA	250	Dimer
CLIC	Animals	$_{35}$CPFS	250	Monomer Dimer Oligomer
GSTL	Terrestrial plants	$_{40}$CPF/YA	230	Monomer
DHAR	Algae, terrestrial plants	$_{20}$CPFC/S	220	Monomer
GHR	Some metazoan but animals, algae, terrestrial plants, fungi, cyanobacteria, bacteria, archaea	$_{50}$CPWA	330	Dimer
mPGES-2	Animals, protists, algae, terrestrial plants	$_{110}$CPFC	310	Dimer
GSTH	Bryophyta, lycophyta	$_{50}$CPF/YT	510	?
GSTI	Algae, bryophyta, lycophyta	$_{120}$CPYC	490	?

The case of CLIC proteins is particular since it exists under both a monomeric soluble and an oligomeric transmembrane form. Moreover, the formation of an intramolecular disulfide bond promotes a structural transition that exposes a large hydrophobic surface changing the monomer into a non-covalent dimer (Littler et al., 2004).

SEQUENCE CHARACTERISTICS AND DOMAIN ORGANIZATION OF CYSTEINYL GSTs

From the phylogenetic tree and the amino acid sequence alignments, there are key sequence differences that allow differentiation of each class. In addition to describing conserved motifs typical of each class, we have paid attention to the presence of N- and C-terminal extensions or of sequence insertions. Some differences are also reflected at the structural level since DHARs and GSTLs are monomeric enzymes, whereas GHRs and mPGES-2s are dimeric enzymes (**Table 2**). Thus, the residues forming the dimeric interface should also constitute a good criterion for distinguishing monomeric from dimeric proteins. This will be discussed further when relevant, either in this section or in the section dealing with the structural characteristics. It is worth noting that N-terminal extensions corresponding to predicted targeting sequences have been excluded when describing the size of the proteins and the percentage identity among isoforms.

From previous phylogenetic analyses conducted with Trx superfamily members, important features to consider have been defined. First, the nature and the position of the active site motif is an essential element. It should be recurrently situated at the beginning of the first α-helix of the thioredoxin fold, which does not mean that extra α-helices cannot be found before. For this reason, the position of the catalytic cysteine can vary although it is generally found around position 20 to 50 (**Table 2**). The

second major characteristic used for comparison is the nature of the residue found before a very conserved *cis*-proline that is typical of proteins of the Trx superfamily. This *cis*-proline is generally positioned 30 to 40 amino acids after the active site signature. According to this feature, an alignment of three representative sequences from each class allowed determining the residues that are conserved among Cys-GSTs (**Figure 3A**). The numbering used is the one corresponding to poplar GSTL1. There are six residues that are mostly conserved in all Cys-GST classes, or at least in the sequences used for the alignment. As expected, the catalytic Cys, found at position 36, and the *cis*-Pro discussed above at position 80, are conserved. Interestingly the Pro37 adjacent to the catalytic Cys is also found in the vast majority of sequences. Hence, the differences between GST classes will be essentially visible by checking the two other positions of the CPxx active site motif. Besides, three other residues are also commonly found in all GSTs. As expected for enzymes that share the same co-substrate, some of the residues contributing to the recognition of GSH, Ser92 and Asp172, are conserved. The last conserved residue is a Gly at position 166 for which function is unknown.

With these features in mind, the difference between classes has been simply analyzed by looking at some specific criteria: (i) the percentage identity, (ii) the size of the proteins, (iii) the presence of extra domains, and (iv) the presence of three specific signatures, i.e. the active site sequence motif and the residues immediately before the *cis*-Pro and before the serine involved in GSH binding. Concerning the first criterion, the percentage identity between members of a given class is usually above 50%, whereas it is usually below 20% between classes.

Protein sizes vary slightly within classes, but they vary more significantly between classes. DHARs and GSTLs are the shorter Cys-GSTs, since they have about the same size ranging from 210 to 220 amino acids for the former and from 230 and 240 amino acids for the latter (**Table 2**). Nevertheless, compared to all other Cys-GSTs, DHARs have a nine amino acid insertion before the α1 helix and thus the active site motif (**Figure 3**). GHRs and mPGES-2s also have approximately the same size, *ca* 330 residues, but the sequence insertions explaining the difference with DHARs or GSTLs are not found at the same position. In the case of mPGES-2, the difference comes from the presence of an N-terminal membrane-anchoring region and of an insertion of about 40 residues between the α4 and α5 helices (**Figure 3B**). This insertion is different from the 20 amino acid insertion found between α3 and α4 helices in vertebrate isoforms which corresponds to two α-helices and two β-sheets (this is further discussed in Section 3D Structures). For GHRs, the size difference is essentially linked to insertions in the Trx domain (*ca* 35 amino acids between the active site motif and the *cis*-Pro, i.e. between the β2 strand and α2 helix) and to a final extension of 20 to 25 amino acids. The latter contains most of the residues responsible for the atypical GHR dimerization (see Section 3D Structures). Finally, GSTIs and GSTHs contain about 500 residues. GSTIs are slightly extended in the N-terminal part, but it is not yet clear whether this is a targeting sequence. Most of the additional sequence (around 120–140 residues) is present at the C-terminus and could correspond to a phycoerythrin α-subunit domain found in phycobilisome proteins (**Figure 3B**). GSTHs are also extended at the C-terminal end but this is due to the presence of an hemerythrin domain of *ca* 150–200 residues as its name suggests, followed by about 100 additional amino acids with no domain annotation (**Figure 3B**).

In the next part, we focused on the conservation of the sequence signatures mentioned above. If we consider the CPYC or CPFC active sites found in glutaredoxins as a reference, all plant Cys-GSTs display a reminiscent catalytic motif that differs by only one residue, with the exception of most GHRs which have two variations in their CPWA motif (**Table 2**). In these proteins, the catalytic cysteine is usually found at position 50. It is interesting to note that most algae have two GHR members including one isoform with an atypical CPWC motif (**Figure 2**). Except two algal sequences having a CPYC active site, mPGES-2s have usually a very conserved CPFC motif found around the position 110 owing to the presence of the N-terminal membrane-anchoring region. For DHARs, except a few sequences where the catalytic cysteine seems to be replaced by a glycine, the active site motif, found around the position 20, is usually quite conserved being of the CPFC or CPFS form. Among GSTLs, the active site motif is found around position 40 and it is mostly of the CP[F/Y]A form. The similarity with GSTOs (active site sequence and size of the proteins) might suggest a common origin. This is further supported by the fact that organisms having GSTOs do not have GSTLs and *vice versa*. Another extremely interesting observation is that GSTLs with SPFA motifs can be found in a few analyzed species as *E. grandis*, *Linum usitatissinum*, *M. domestica*, and *Ricinus communis*. This is also true for some fungal GSTOs found in particular in *Phanerochaete chrysosporium* or *carnosa* and *Trametes versicolor* where the classical CPY/FA motif is replaced by a SPY[C/S] motif (Morel et al., 2009). Although it should confer opposite properties (glutathionylating vs. deglutathionylating activities) to the proteins, this suggests that GST genes can be maintained in genomes as long as the replaced amino acid conserves catalytic functions. Concerning GSTIs and GSTHs, the fact that these sequences are restricted to a few species and that the number of sequences available is low makes the analysis of amino acid conservation less robust. Nevertheless, it appears that most GSTI sequences exhibit a conserved WCPYC motif, except one representative from *C. reinhardtii* that has a RCPYC sequence. If it turns out that the N-terminal extension is indeed a targeting sequence, this motif is located around the position 60, otherwise it is located around the position 120. In GSTHs, the active sequence is CP[F/Y]T and depending on the isoform considered, it is usually found around position 50 or 70.

Finally, the residues associated to the *cis*-Pro80 and to Ser92 may help to definitely discriminate GST classes. The classes that cannot be differentiated using these signatures are GSTLs and GHRs which usually exhibit VP and ES motives, and DHARs and mPGES-2 which have VP and DS motives. However, as explained above, the other factors will allow distinguishing them. The last two classes, GSTIs and GSTHs, have specific recognizable sequences, LP and ES or [F/Y]P and GS, respectively.

GENE EXPRESSION

To date, there is not much data available on plant Cys-GSTs, both at genetic and physiological levels. Nonetheless, the physiological

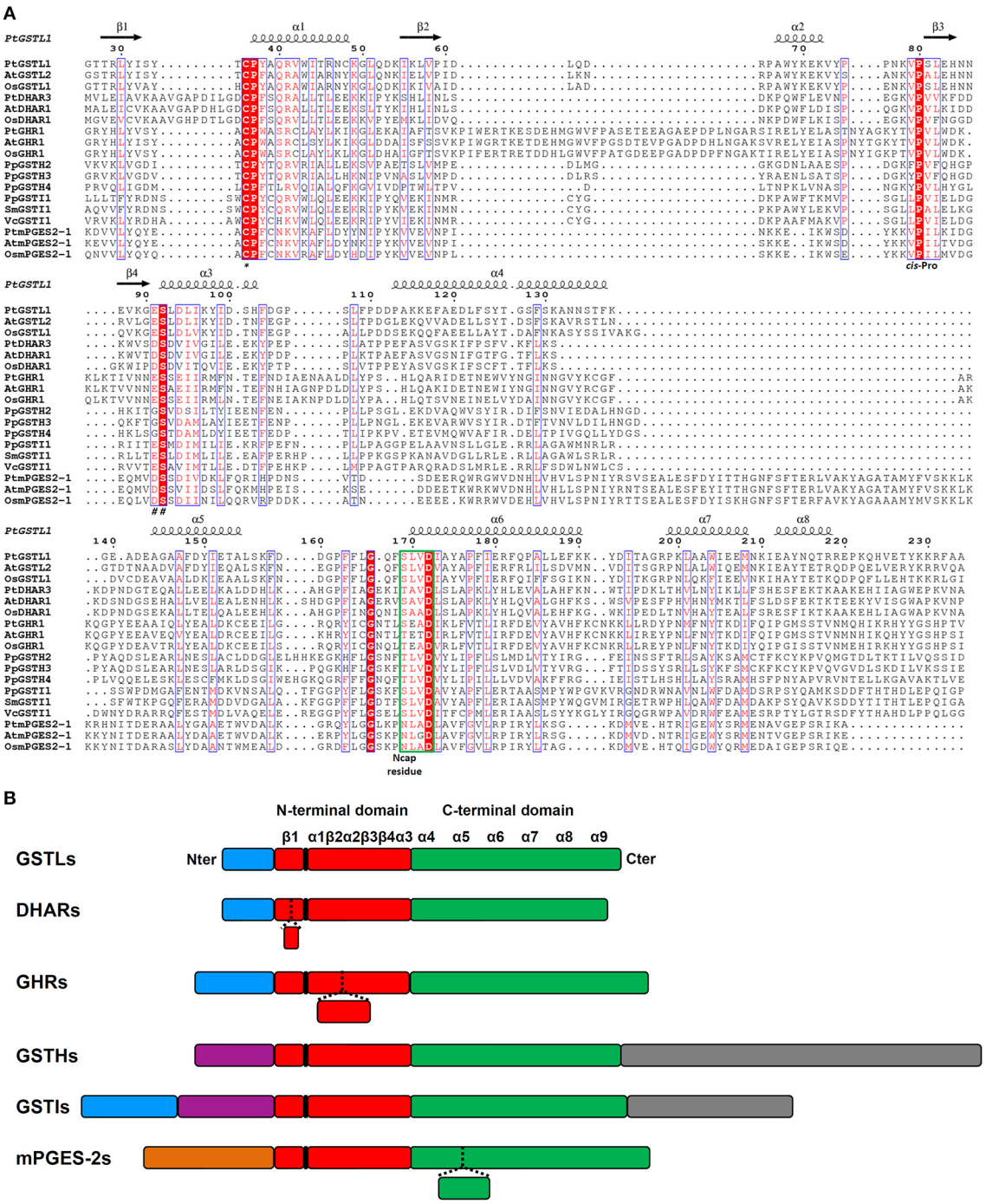

FIGURE 3 | Amino acid alignment and protein architecture of plant Cys-GSTs. (A) Amino acid sequence alignment of three representative members from each Cys-GST class. The sequences were structurally aligned using PROMALS3D server using as references the solved structures of PtGSTL1 [PDB code 4PQH (Lallement et al., 2014)], PtGSTL3 [PDB code 4PQI (Lallement et al., 2014)], *Phanerochaete chrysosporium* GHR1 [PDB code 3PPU (Meux et al., 2011)], and *Macaca fascicularis* mPGES-2 [PDB code 1Z9H (Yamada et al., 2005)] since there is no structure available for DHARs, GSTIs, and GSTHs. Since the structure of poplar GSTL1 has been solved, its secondary structures have been indicated as reference using ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/index.php), with the helices and the arrows corresponding respectively to α-helices and to β-strands. Strictly conserved residues are marked in white characters on a red background, whereas residues with similar functional groups are in red characters on white background. The indicated numbering corresponds to that of PtGSTL1 which has been used as a whole. For clarity, N- and C-terminal extensions present in Cys-GSTs have been removed from the alignment to keep only the sequences corresponding to secondary structures forming the GST fold. At is for *Arabidopsis thaliana*, Pt for *Populus trichocarpa*, Os for *Oryza sativa*, Pp for *Physcomitrella patens*, Sm for *Selaginella moellendorffii*, and Vc for *Volvox carteri*. The catalytic cysteine (*), *cis*-proline (*cis*-Pro), residues stabilizing the γ-glutamate residue of glutathione (##) and N-cap residue are shown. The N-capping box is surrounded in green. **(B)** Schematic representation of the

(Continued)

FIGURE 3 | Continued
protein architecture of plant Cys-GSTs. The N-terminal Trx-like domain and the all-helical C-terminal domain are represented respectively in red and green. Blue boxes correspond to putative or confirmed targeting sequences. The orange box corresponds to the membrane anchoring tail of mPGES-2. Purple boxes represent N-terminal extensions that do not correspond to targeting sequences and gray boxes represent additional C-terminal domains. The position of the active site motif harboring the catalytic cysteine is indicated in black. The presence of inserted sequences in some classes corresponds to dashed lines in other classes. Secondary structures are shown as α-helices and β-strands. The size of the boxes is proportional to the length in amino acids.

roles of these enzymes can certainly be better understood by delineating the gene expression in plant organs or in response to environmental constraints. Since only partial information is available for each class, analyzing microarray experiments represents a valuable approach ahead of targeted expression studies. Hereto, *A. thaliana* microarray experiments were analyzed to assess both the developmental expression patterns of each GST using the EFP browser (Winter et al., 2007) and the transcriptional regulation occurring in response to environmental stimuli using Genevestigator (3072 perturbations were analyzed) (Hruz et al., 2008). Among the 14 genes coding for Cys-GSTs in Arabidopsis, four groups can be distinguished based on the absolute levels of expression, ranging from high to low: (i) DHAR1, DHAR3 and GSTL3, (ii) GSTL2, DHAR2 and GHR1, (iii) mPGES-2, GHR2 and GHR4, and (iv) GSTL1 and GHR3.

The expression of mPGES-2 genes has never been studied in plants. In mammals, however, the genes and corresponding enzymes have been shown to be constitutively expressed and involved in prostaglandin E metabolism, respectively (Murakami et al., 2003). In Arabidopsis, *mPGES-2* transcripts are found in all analyzed organs and at quite constant level (**Figure 4**). Based on the data available in Genevestigator, *A. thaliana mPGES-2* is the most significantly regulated gene in response to environmental stresses among Cys-GSTs. It is overexpressed in response to cold, drought, hypoxia, increases in light, chemical and biotic stresses, and shows its strongest up-regulation under heat stress. Altogether, this suggests that this gene may be involved in general tolerance mechanisms to biotic and abiotic stresses.

Regarding *GSTL* genes, *AtGSTL2* and *AtGSTL3* show similar expression patterns, particularly in green tissues, such as leaves, flower sepals, siliques, and developing seeds, whereas *AtGSTL1* transcripts are almost exclusively found in roots, senescing leaves, and pollen (**Figure 4**). In terms of stress responses, *AtGSTL1* is strongly up-regulated in several conditions including biotic interactions, treatment with chemicals, salt and iron-starvation stresses and, to a lesser extent, in response to hypoxia. This is consistent with a study showing that the *AtGSTL1* gene is induced in root cell cultures in response to buthionine sulfoximine (BSO), tert-butyl hydroperoxide, dichlormid, and 2,4 dichlorophenoxy acetic acid (Dixon et al., 2002). This also corroborates the observation that expression of tomato *GSTL3* is induced by salt treatments in both roots and leaves (Csiszar et al., 2014). Interestingly, in contrast to *AtGSTL1*, *AtGSTL2*, and *AtGSTL3* are less responsive to environmental stress factors, but respond specifically to conditions involving increases in light, such as germination and light-shifts. These differences in expression patterns may help identifying the function of the three *AtGSTL* genes. Additionally, a number of studies has explored the expression and tissue distribution of *GSTLs* in other plant species. For instance, the three rice *GSTL* genes are all differentially expressed in response to arsenic treatments (Kumar et al., 2013a). Moreover, *OsGSTL1* and *OsGSTL2* are both constitutively expressed and involved in xenobiotic and oxidative stress tolerance in rice, whereas *OsGSTL2* is also specifically up-regulated in roots after herbicide (chlorsulfuron and glyphosate) and hormone treatments (salicylic acid and naphthalene acetic acid) (Hu et al., 2009, 2011a,b). Consistently, Arabidopsis transgenic lines expressing *OsGSTL2* are more tolerant to abiotic stresses such as heavy metals, cold, drought, and salt stress (Kumar et al., 2013a,b). Recently, the expression of the three poplar GSTL genes was studied in a naturally growing *Populus trichocarpa* adult tree (Lallement et al., 2014). One of these genes, *PtGSTL3*, generates two transcripts by alternative splicing, *PtGSTL3A* and *PtGSTL3B*, the latter being very weakly expressed. While *PtGSTL2* and *PtGSTL3A* seem to be constitutively expressed, all PtGSTL genes are preferentially expressed in the reproductive organs (flowers, fruits, buds) (Lallement et al., 2014). Nevertheless, poplar *GSTLs* have also been detected in leaves and roots (Lan et al., 2009). Altogether these results suggest that *GSTLs* are mainly expressed in organs that have a more intense secondary metabolism, which is consistent with the proposal that GSTLs are involved in the biosynthesis and/or maintenance of the flavonoid pool (see the Section Enzymatic Properties and Physiological Roles).

The three *A. thaliana DHAR* genes are expressed in most organs tested. Although *AtDHAR1* and *AtDHAR3* are either weakly or not expressed in pollen, this may be compensated by *AtDHAR2* which has its highest expression in this organ (**Figure 4**). Aside from this, the only notable difference is that *AtDHAR3* is relatively strongly expressed in leaves, which is consistent with its predicted localization to plastids (**Figure 4**). In other organisms where *DHAR* expression was studied, the genes were shown to be expressed in most tissues/organs. This is the case for the three poplar *DHARs* in roots, shoots, leaves, phloem, and buds (Lan et al., 2009; Tang and Yang, 2013) and for one *DHAR* from *Pinus bungeana* in buds, needles, phloem from stems, roots, and seedlings (Yang et al., 2009). However, in the moss *P. patens*, one of the three genes does not seem to be expressed at all (Liu et al., 2013). In response to environmental variations, the microarray data of *A. thaliana* indicates that *AtDHAR2* is the most responsive gene being up-regulated during germination and in response to biotic stress, chemicals, hypoxia, and drought. In contrast, *AtDHAR1* and *AtDHAR3* are up-regulated in only two conditions, excess light or drought and excess light or germination, respectively. Surprisingly, both genes are down-regulated in response to chemicals, a condition where GSTs are usually over-expressed. In plants, several independent studies have been performed with the aim of addressing the role of *DHARs* during stress response. For instance, *AtDHAR1* is

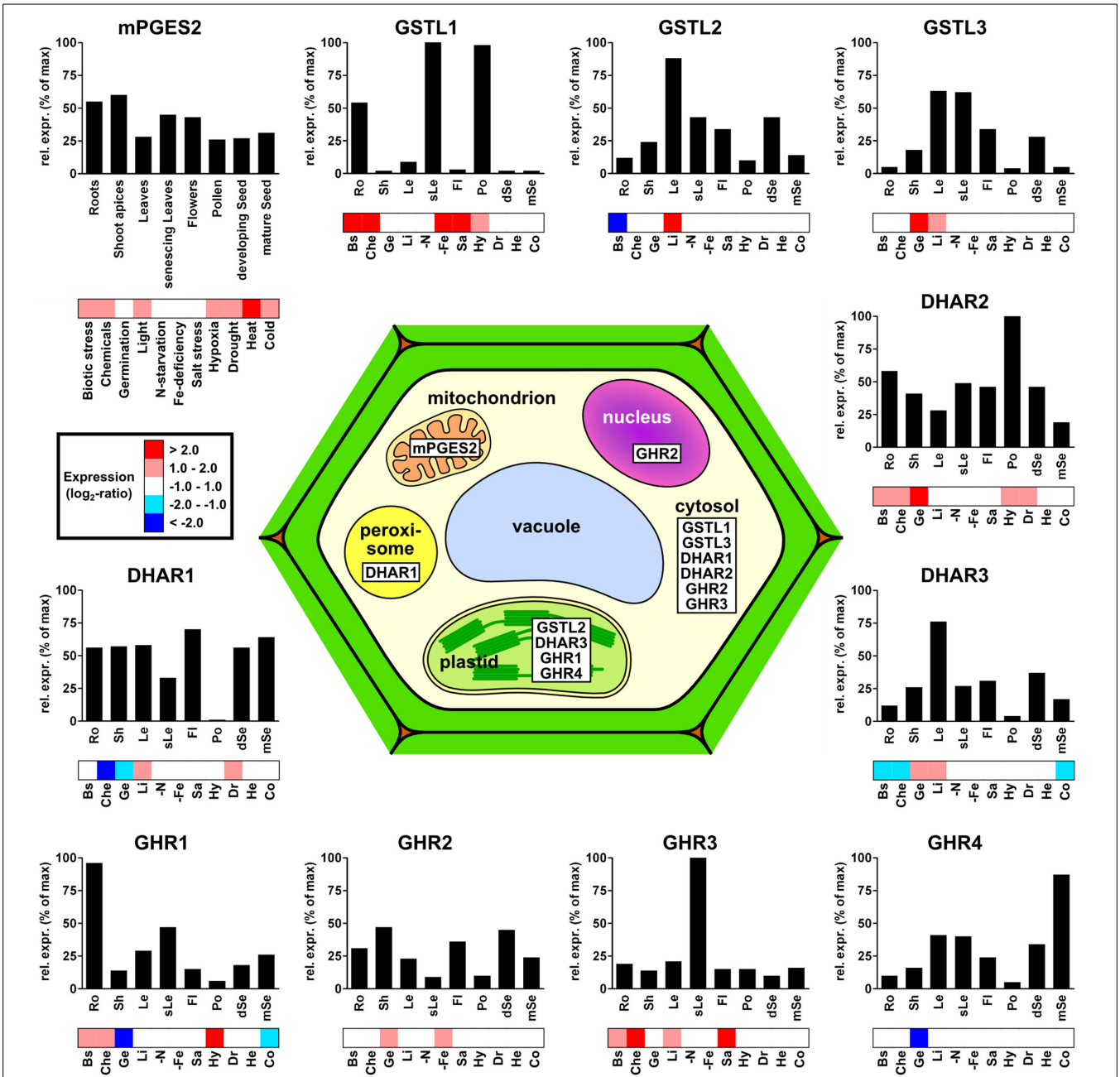

FIGURE 4 | Subcellular localization and gene expression profiles of the different *Arabidopsis thaliana* Cys-GSTs. For each GST, the relative transcript expression is shown in relation to both plant developmental stages (bar-graphs) and perturbations (heatmaps). For clarity, the number of developmental conditions was reduced to eight classes, where each gene was normalized to its maximum expression within the selected dataset (see Materials and Methods). For the response to perturbations, the expression values were organized into five color-coded groups based on their $\log_2$-ratios. For clarity, the numerous perturbations included in the dataset were grouped into 11 classes: Biotic stress (Bs), Chemicals (Che), Germination (Ge), Light (Li), N-starvation (-N), Fe-deficiency (-Fe), Salt stress (Sa), Hypoxia (Hy), Drought (Dr), Heat (He), and Cold (Co).

up-regulated in response to norflurazon, menadione, paraquat, and antimycin A, treatments known to produce reactive oxygen species (Chew et al., 2003). On the other hand, *AtDHAR2* expression is induced in root cell cultures in response to BSO and chloro-dinitrobenzene (CDNB) (Dixon et al., 2002). However, in other organisms, *DHARs* are not always regulated in the same manner in response to stress conditions. For example, *DHAR2* from *P. patens* is strongly down-regulated by the addition of H_2O_2, salt, salicylic acid, and atrazine (Liu et al., 2013), whereas poplar *DHAR2*, in contrast to poplar *DHAR3*, is up-regulated in shoots but not in roots in response to H_2O_2, atrazine and to a lesser extent CDNB (Lan et al., 2009). Altogether these data

point to a crucial function of DHARs and consequently ascorbic acid for stress responses, although the pattern of expression and regulation of DHAR genes differ from one organism to another.

Since GHRs were only recently identified in plants, they have not been studied in detail and very little is known about their expression and regulation. In yeast, GHR genes were formerly referred to as GSTO1, GSTO2, and GSTO3. The *GSTO1* gene, which encodes a peroxisomal protein involved in sulfur metabolism, was shown to be induced by oxidative stress conditions (Barreto et al., 2006). Based on the analysis of *A. thaliana* microarrays, it is clear that GHRs are among the Cys-GSTs that are the least expressed, even though they are expressed in all plant organs analyzed. Apart from *GHR2*, which shows no preferential expression, the genes are each predominantly expressed in a particular organ, roots for *GHR1*, senescent leaves for *GHR3* and mature seeds for *GHR4* (**Figure 4**). In response to stress conditions, *A. thaliana* GHR genes are differentially regulated as well. *AtGHR4* appears only to be down-regulated during germination. While *AtGHR1* is down-regulated during germination and in response to cold, it is up-regulated in response to chemicals and biotic stress, together with *GHR3*. However, the expression of *AtGHR1* is also increased by hypoxia, while *AtGHR3* is increased in response to salt treatments and increases in light. Finally, in contrast to *AtGHR1* and *AtGHR4*, *AtGHR2* is up-regulated during germination and also shows up-regulation in response to iron starvation.

SUBCELLULAR LOCALIZATION

Deciphering the subcellular localization of all these proteins should also contribute to the understanding of their biological role. The data present in the literature for *A. thaliana* and poplar Cys-GSTs, originating from proteomic studies or from GFP fusion experiments have been compiled together with bioinformatic predictions for the presence of targeting sequences and summarized in **Figure 4** and **Table 3**. First, while not much is known about plant mPGES-2s, mammalian mPGES-2s exhibit a dual subcellular localization associated to both the Golgi membrane through their N-terminal part and the cytoplasm after proteolytic cleavage of the N-terminal hydrophobic domain (Tanikawa et al., 2002; Murakami et al., 2003). Similarly, plant mPGES-2 proteins possess an N-terminal extension, but it is predicted to correspond to a mitochondrial or plastid targeting sequence. This might be supported by the identification of this protein in two proteomic studies of mitochondrial protein fractions (**Table 3**) (Heazlewood et al., 2004; Klodmann et al., 2011). However, a careful inspection of the nature of the amino acids present in this region is rather consistent with a membrane-anchoring tail. Accordingly, the Arabidopsis ortholog was also identified in a proteomic study of plasma membrane proteins and an N-terminal transmembrane domain is indeed predicted by some prediction programs devoted to their identification (**Table 3**) (Mitra et al., 2009). Altogether, these data will have to be firmly established by complementary experiments, especially if a cleavage could also generate soluble isoforms. The mPGES-2s could be the only membrane associated Cys-GSTs since no other protein was predicted to possess a membrane-anchoring region.

Among DHARs, based on its occurrence in chloroplast proteome analyses and the presence of an N-terminal extension in the protein sequence, *A. thaliana* DHAR3 should be chloroplastic (**Table 3**). Poplar and *P. patens* orthologs have a similar localization (Liu et al., 2013; Tang and Yang, 2013). The two other proteins from Arabidopsis are predicted to be cytosolic as they do not exhibit visible targeting sequences. However, proteome analyses and YFP fusion proteins indicate that AtDHAR1 is also present in peroxisomes (Reumann et al., 2009; Grefen et al., 2010).

Concerning GSTLs, based on proteome analyses and on the presence of an N-terminal predicted targeting sequence, AtGSTL2 is clearly a plastidial protein and it may also be present in peroxisomes as shown by GFP fusion experiments as well as in the cytosol (Dixon et al., 2009). The poplar ortholog, PtGSTL1, is also present in plastids but *a priori* not in peroxisomes (Lallement et al., 2014) and the *P. patens* isoform is also plastidial (Liu et al., 2013). The two other GSTLs found in *A. thaliana* and *P. trichocarpa* should be cytosolic proteins, although a nuclear localization was observed when poplar proteins were fused to GFP (Lallement et al., 2014). Considering the absence of a clear NLS (nuclear localization signal), this nuclear localization is more likely due to a passive diffusion through nuclear pore rather than to a specific targeting.

Concerning GHRs, there is no information available yet. Among the four isoforms found in *A. thaliana*, two of them are predicted to be chloroplastic proteins and have additionally been indeed identified from high-throughput proteomic analyses (**Table 3**). Concerning Hemerythrin GSTs, the proteins do not exhibit clear targeting sequences suggesting cytosolic localization. Accordingly, four GSTHs from *P. patens*, PpGSTH1, 2, 3, and 7, presented a nucleo-cytoplasmic localization in GFP fusion experiments (Liu et al., 2013). Concerning GSTIs, according to the existence of clearly visible N-terminal extensions in some representative members, several prediction programs indicate that they could be targeted either to the chloroplasts or to mitochondria, although this remains to be demonstrated experimentally.

3D STRUCTURES

At the structural level, GSTs consist of an N-terminal domain adopting a thioredoxin fold and an all-helical C-terminal domain (Atkinson and Babbitt, 2009). The GSH binding site, or G site, is located in a cleft formed between the two domains and most of the residues contacting GSH are provided by the N-terminal domain. The binding site for the hydrophobic electrophiles, or H site, is located immediately adjacent to the G site and forms part of the solvent-exposed cleft between both domains. For the H site, most of the residues contacting the electrophiles are provided by the C-terminal domain. Both sites form the protein active site. Moreover, non-catalytic ligandin sites (L site) were defined in GSTs. Two types of L site may be roughly distinguished: those overlapping partially with the H site and those located at the dimer interface straddling the two fold axis (Litwack et al., 1971; McTigue et al., 1995; Rossjohn et al., 1998; Smith et al., 2003; Axarli et al., 2004; Dixon et al., 2008; Brock et al., 2013).

The N-terminal thioredoxin domain is often described as two distinct motifs: an N-terminal motif ($\beta1\alpha1\beta2$) and a C-terminal

Table 3 | Subcellular localization of *Arabidopsis thaliana* Cys-GST members and of poplar orthologs.

Gene name	Accession number	Predicted subcellular localization	Confirmed localization and other proteomic evidence	Amino acid length	Orthologs in poplar	References
DHAR1	At1g19570	Elsewhere	- *FP fusions:* cytosol[a], peroxisome[b] - *High-throughput proteomic:* mitochondria[c], cytosol[d], plasma membrane[e], chloroplast[f]	213	- *FP fusions:* DHAR2: cytosol[b], DHAR3: cytosol[b]	[a]Grefen et al., 2010 [b]Reumann et al., 2009 [c]Chew et al., 2003 [d]Ito et al., 2011 [e]Marmagne et al., 2007 [f]Peltier et al., 2006
DHAR2	At1g75270	Elsewhere	- *High-throughput proteomic*: cytosol[a], plasma membrane[b]	213	- *FP fusions:* DHAR2: cytosol[b], DHAR3: cytosol[b]	[a]Ito et al., 2011 [b]Marmagne et al., 2007 [c]Tang and Yang, 2013
DHAR3	At5g16710	Chloroplast	- *High-throughput proteomic*: chloroplast[a]	258	- *FP fusions:* DHAR1: chloroplast[b]	[a]Zybailov et al., 2008 [b]Tang and Yang, 2013
GSTL1	At5g02780	Elsewhere	None	237	- *FP fusions:* GSTL2, GSTL3A & B: nucleocytoplasmic[a]	[a]Lallement et al., 2014
GSTL2	At3g55040	Chloroplast	- *FP fusions*: peroxisome[a], - *High-throughput proteomic*: chloroplast[b–d]	292	- *FP fusions:* GSTL1: chloroplast[e]	[a]Dixon et al., 2009 [b]Zybailov et al., 2008 [c]Peltier et al., 2006 [d]Ferro et al., 2010 [e]Lallement et al., 2014
GSTL3	At5g02790	Elsewhere	- *High-throughput proteomic*: cytosol[a]	235	- *FP fusions:* GSTL2, GSTL3A & B: nucleocytoplasmic[b]	[a]Ito et al., 2011 [b]Lallement et al., 2014
GHR1	At4g19880	Chloroplast	- *High-throughput proteomic*: cytosol[a], chloroplast[b]	356		[a]Ito et al., 2011 [b]Klodmann et al., 2011
GHR2	At5g45020	Elsewhere	None	325		
GHR3	At5g44990	Elsewhere	None	350		
GHR4	At5g44000	Chloroplast	- *High-throughput proteomic*: chloroplast[a]	399		[a]Ferro et al., 2010
mPGES-2	At5g42150	Mitochondria or chloroplast	- *High-throughput proteomic*: mitochondria[a,b], plasma membrane[c]	315		[a]Heazlewood et al., 2004 [b]Klodmann et al., 2011 [c]Mitra et al., 2009

The prediction of subcellular localization was performed by compiling results obtained from various softwares as Predotar, TargetP, and Wolfpsort. Experimental confirmation consisting of high-throughput proteome analyses and fusions with fluorescent proteins (FP fusions) and associated references are indicated when available.

motif (β3β4α3) linked by helix α2 and which together form a four β-sheet in the order 2134 with β3 anti-parallel to the others (**Figure 5A**). Despite the low primary sequence conservation between GST classes, the position of the key residues is maintained. As mentioned above, the cysteine or serine of the catalytic signature is located at the beginning of α1 helix which also contains charged residues involved in the proton transfer reaction. For GSTs having a catalytic tyrosine residue, it is positioned at the end of the β1 strand. The invariant *cis*-Pro residue is located in the loop region before β3 and is thought to be implicated in the maintenance of the enzyme fold rather than playing a role in the enzymatic reaction (**Figure 3A**) (Allocati et al., 1999). The residues responsible for the non-covalent anchoring of GSH are well-conserved in most known GSTs. Glutamate/aspartate/glutamine residues and the adjacent serine residue in the loop β4-α3, stabilize the charged group of GluGS (γ-glutamate residue of glutathione). The main chain of a valine/leucine/isoleucine/threonine residue that precedes the conserved *cis*-proline is hydrogen-bonded to the backbone of CysGS (cysteine residue of glutathione) (**Figure 3A**). In the loop β2-α2, a charged residue (lysine, arginine), not present in all GSTs, makes a salt bridge with the carboxyl group of GlyGS (glycine residue of glutathione). In addition to these usual interactions with GSH, other less conserved residues can also contribute to the stabilization of GSH in specific cases.

The C-terminal domain exhibits a bundle of helices whose number varies between each class. This less conserved domain, compared to the N-terminal domain, notably contains a well-conserved N-capping box (S/TXXD) including the S/T N-cap residue (**Figure 3A**) and a hydrophobic staple motif located at the

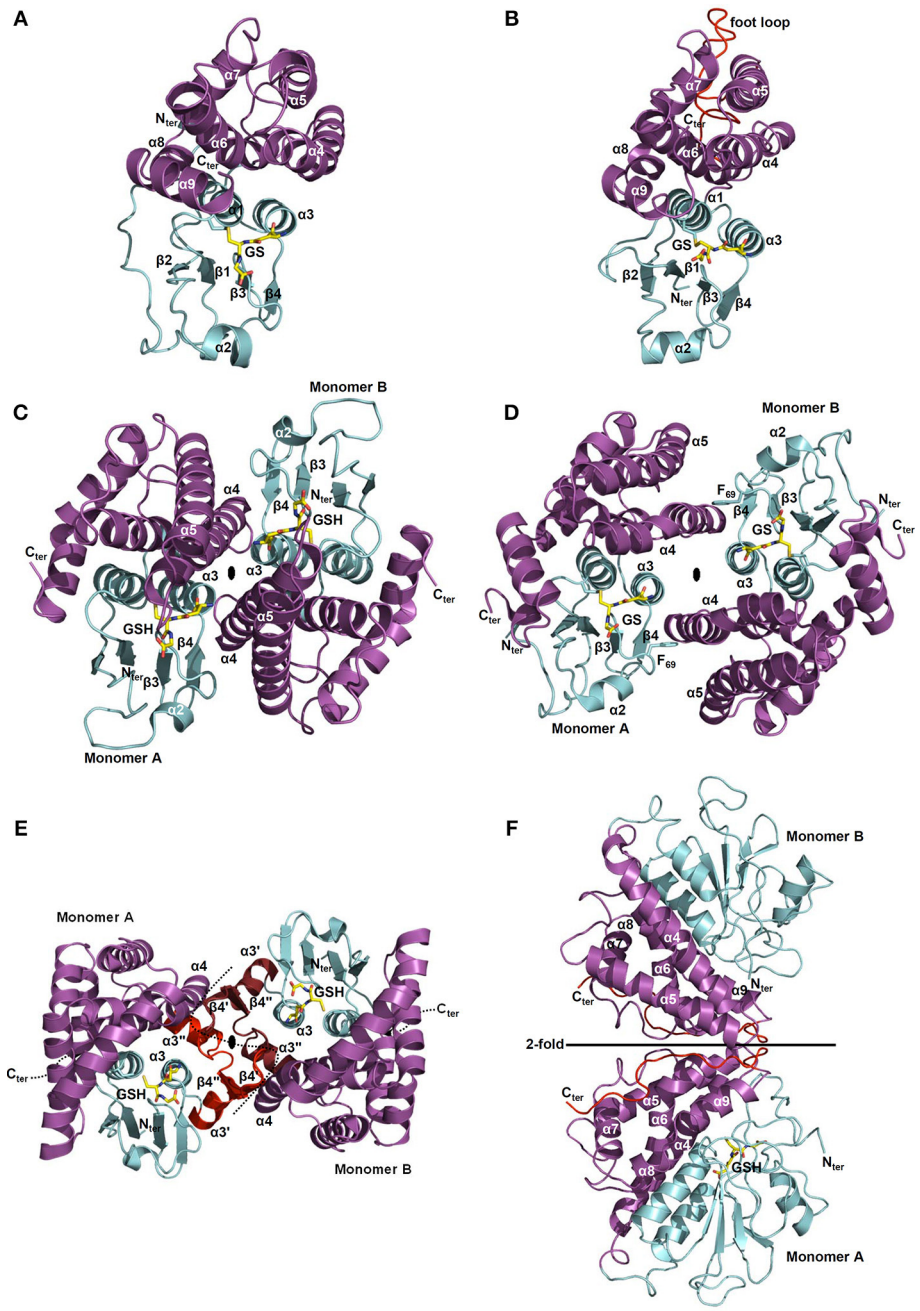

FIGURE 5 | Structural organization of Cys-GSTs. All structures are shown as cartoon with the N- and C-terminal domains colored in cyan and in purple, respectively. Glutathione (GSH) or glutathione adducts (GS) are represented as sticks. In **F**, glutathione is only present in monomer A. All figures have been prepared with Pymol software. **(A,B)** Monomeric organization of **(A)** GSTL3 from *Populus trichocarpa* (PDB code 4PQI) and **(B)** CLIC1 from *Homo sapiens* (PDB code 1K0M). These monomeric enzymes illustrate the classical GST fold which consists of an N-terminal domain adopting a thioredoxin fold (β1α1β2α2β3β4α3) and an all helical C-terminal domain. Human CLIC1 **(B)** harbors a long negatively charged loop also referred as "foot loop" (colored in red) inserted between helices 5 and 6. This loop is characteristic of CLICs and might be responsible for interaction with other proteins. The glutathione adduct (GS) has been modeled based on the superimposition with a glutathionylated version of *Homo sapiens* CLIC1 (PDB code 1K0N). **(C,D)** Classical dimerization mode of GSTs as shown using **(C)** *Ochrobactrum anthropi* GSTB (PDB code 2NTO) and **(D)** *Homo sapiens* GSTO1-1 (PDB code 1EEM). The monomers associate along a structural C2 axis. The N-terminal domain (loop α2-β3, strand β4 and helix α3) of one subunit interacts with the C-terminal domain (helices α4 and α5) of the other monomer. The dimer interface is either hydrophilic **(C)** or hydrophobic **(D)**. The hydrophobic interaction is characterized by the insertion of a phenylalanine (or a tyrosine) residue belonging to the α2-β3 loop into a hydrophobic pocket located between helices α4 and α5 of the C-terminal domain of the other subunit ("lock-and-key" motif). **(E)** *Macaca fascicularis* mPGES-2 (PDB code 2PBJ). The dimerization occurs via a α3′β4′β4″α3″ structure (colored in red) inserted between α3 and α4 that interacts with those of the other monomer (colored in ruby). Note that this insertion is not found in plant sequences. **(F)** *Phanerochaete chrysosporium* GHR1 (3PPU). The two monomers interact via their C-terminal domain (in red) and are related to each other by a 2-fold symmetry axis.

N-terminal part of α6 helix. The N-capping box motif has been proposed to participate to the nucleation of helices as well as their folding and stabilization by forming reciprocal main chain-side chain hydrogen bonds between the N-cap (Ser/Thr) and the N3 (Glu/Asp) residues. The hydrophobic staple motif consists of a specific i,i+5 hydrophobic interaction between a residue (N′) that precedes the N-cap residue and a residue (N4) located within the α6-helix. The nomenclature commonly used is as follows: N-N′-Ncap-N1-N2-N3-N4 (XhS/TXXDh, with h: hydrophobic residue and X: non conserved residue) (Richardson and Richardson, 1988). These two local structural motifs have a critical role in protein folding and stability of α-helices. The substitution of the capping residue greatly destabilizes the structure of GSTs, as well as their folding. It has also been proposed that the hydrophobic staple motif represents an evolutionarily conserved determinant for rapid folding of the enzyme. In addition, a glycine residue located four amino acids upstream (three residues in GSTLs) the N-cap residue (S/T) is also well-conserved in GSTs and is likely essential for folding by stabilizing the GXXh(S/T)XXDh conserved loop-helix substructure (Kong et al., 2003) demonstrating the importance of these motifs both for protein folding and stability. Concerning the residues involved in the H site, they are generally hydrophobic and are located in a crevice between the N- and C-terminal domains at the vicinity of the G site. The nature of the amino acids contributing to the substrate recognition in this H-site has not been identified so often since it generally requires the crystal structure of complexes. Moreover, from known examples, they are quite variable among GST classes which likely explain the diversity of substrates accommodated by the different GSTs but may at the same time also explain the lack of specificity among certain classes for some substrates (Wilce and Parker, 1994; Armstrong, 1997). For these reasons, we will not discuss in detail the structure and residues forming the H site in each GST class.

With the exception of a few classes such as GSTLs, DHARs and soluble CLICs that exist as monomeric enzymes (**Figures 5A,B**) (Dixon et al., 2002), other GSTs are mostly dimeric proteins and very often adopt the same dimerization mode. Both subunits are connected along a structural C2 axis roughly parallel to helix bundle axis (binary axial symmetry). The main interactions between the two subunits are held between the N-terminal domain of one subunit and the C-terminal domain of the other. Thus, the loop α2-β3, the strand β4 and the helix α3 of one subunit interacts with the helices α4 and α5 of the other subunit as in GSTBs (**Figure 5C**). This dimer is considered as the classical dimerization mode in GSTs. In Theta, Sigma and Beta members, the interaction surface is rather hydrophilic whereas in Alpha, Phi, Mu, Omega, Pi, Tau, Zeta, and FuA GSTs, the surface is more hydrophobic (Frova, 2006). The hydrophobic interaction is characterized by a hydrophobic "lock-and-key" (or "ball and socket") motif which holds the two protomers together and which is established due to the side chain of a phenylalanine (or a tyrosine) residue (key) belonging to the α2-β3 loop (Dirr et al., 1994). This residue is inserted into a hydrophobic pocket (lock) located between helices α4 and α5 of the C-terminal domain of the other subunit as shown for human GSTO1-1 (**Figure 5D**). This particular interaction is absent in Theta, Sigma, Beta, and Tau members and is replaced by an extensive network of polar interactions (**Figure 5C**) (Armstrong, 1997; Stevens et al., 2000). Beyond the canonical dimer, other dimerization modes have been described for GSTs. For example, FuA GST dimeric arrangement is close to the one observed in the canonical dimer in that their C2 axis is along the same direction (Figure S1A). In FuA GSTs, the two protomers are translated in the interface plane bringing the α-helical domains closer to each other. An additional β-hairpin (β2′-β2″) inserted between α2 and β3 inhibits the formation of the regular GST dimer and acts as a lid over the G site (Mathieu et al., 2012). A GST from the soil bacterium *Ralstonia solanacearum* (PDB code 4KF9) exhibits a similar dimerization mode as GSTFuA. In this case, the β-hairpin (β2′-β2″) is absent but a long C-terminal extension, which extends the β-sheet structure, prevents the formation of the classical dimer (Figure S1B). In *Macaca fascicularis* mPGES-2, the dimerization remains similar to the canonical assembly and occurs through an insertion of two α-helices and two β-strands (α3′β4′β4″α3″) between α3 and α4 that interacts with those of the other monomer (**Figure 5E**) (Yamada et al., 2005; Yamada and Takusagawa, 2007). However, this insertion seems to be specific to vertebrates and is absent in photosynthetic organisms, which suggests a different organization. In GHRs, the mode of dimerization is completely different, the monomers associate exclusively via their C-terminal domain and notably via a coil of about 20 residues that follows the helix α9 (Meux et al., 2011). Helix α9 is a structural characteristic that is also found in GSTOs (Board et al., 2000), Tau GSTs (Thom et al., 2002), Delta GSTs (Oakley et al., 2001) and GSTLs (Lallement et al., 2014). The 20 C-terminal residues of one monomer mainly interact with the N-terminal end of helix α5 and with the C-terminal end of helix α6 of the other monomer, allowing the formation of a dimer that completely differs from the usual GST dimer (**Figure 5F**) (Meux et al., 2011). In addition, a recently characterized GST from *Leishmania infantum* (TDR1 protein) does not exhibit the canonical dimerization mode but consists of a unique trimer of subunits each containing two glutathione S-transferase domains (Figure S1C) (Fyfe et al., 2012). While the diversity of GST quaternary structures might still grow with the release and accumulation of structural data, the majority of GSTs adopts the canonical dimeric quaternary structure.

To date, there are 10 structures of bacterial GSTBs (**Table 4**). A *B. xenovorans* GSTB structure has been obtained in complex with GSH in the G site and the physiological product, 2-hydroxy-6-oxo-6-phenyl-2,4-dienoate, in the H site (Tocheva et al., 2006). Concerning CLICs, structures from three organisms, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* are available (Harrop et al., 2001; Littler et al., 2008). Besides, a few structures have been obtained for the other cysteinyl-GSTs but not for DHAR. For mPGES-2s, only the structure of the *M. fascicularis* isoform has been solved (Yamada et al., 2005). Concerning GSTOs, in addition to one structure from *Bombyx mori* GSTO3 (Chen et al., 2011), several structures are known for human GSTO1 and GSTO2, alone or in complex with GSH or some substrates (**Table 4**). Recently, the first 3D structures of GSTLs (poplar GSTL1 and L3) in complex with glutathione have been solved (Lallement et al., 2014). Finally, a few GHR/xi GST structures have been solved from various

Table 4 | Tridimensional structures of Cys-GSTs from all kingdoms.

Class	Name	Organism	Ligand 1	Ligand 2	PDB	References
?	TDR1	*L. infantum*	GSH	1,2-Ethanediol	4AGS	Fyfe et al., 2012
?	LigG	*S. paucimobilis*	GSH	SO_4^{2-}; Acetate	4G10	Meux et al., 2012
GSTB	BphK	*B. xenovorans*	GSH	2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid	2DSA	Tocheva et al., 2006
GSTB		*E. coli*	GTS	–	1A0F	Nishida et al., 1998
GSTB		*M. haemolytica*	GSH	Triethylene glycol; Cl^-; Acetate	4IW9	Unpublished
GSTB		*M. capsulatus*	GSH	Glycerol	3UAR	Unpublished
GSTB		*O. anthropi*	GSH	SO_4^{2-}	2NTO	Federici et al., 2007
GSTB		*P. mirabilis*	GSH	–	1PMT	Rossjohn et al., 1998
GSTB		*S. flexneri*	GSH	–	4KGI	Unpublished
GSTB		*S. paucimobilis*	GSH	–	1F2E	Unpublished
GSTB		*X. fastidiosa*	GSH	Cl^-	2X64	Unpublished
GSTB		*Y. pestis*	GSH	Glycerol	4G9H	Unpublished
CLIC	EXC-4	*C. elegans*	–	Ca^{2+}	2YV9	Littler et al., 2008
CLIC		*D. melanogaster*	–	Ca^{2+}; I^-	2YV7	Littler et al., 2008
CLIC	CLIC1	*H. sapiens*	GSH	–	1K0N	Harrop et al., 2001
CLIC	CLIC4	*H. sapiens*	–	–	2AHE	Littler et al., 2004
CLIC	CLIC2	*H. sapiens*	GSH	–	2R4V	Cromer et al., 2007
CLIC	CLIC3	*H. sapiens*	–	SO_4^{2-}	3FY7	Littler et al., 2010
GRX	Grx2	*E. coli*	–	–	1G7O	Xia et al., 2001
GRX	Grx2	*S. enterica*	GSH	SO_4^{2-}; Cl^-	3IR4	Unpublished
GSTO*	GSTO3-3	*B. mori*	–	Glycerol	3RBT	Chen et al., 2011
GSTO	GSTO1-1	*H. sapiens*	GSH	SO_4^{2-}	1EEM	Board et al., 2000
GSTO	GSTO2-2	*H. sapiens*	GSH	Cl^-	3Q19	Zhou et al., 2012
mPGES-2		*M. fascicularis*	–	Indomethacin; Cl^-; Acetate	1Z9H	Yamada et al., 2005
GHR	YqjG	*E. coli*	–	GS-menadione	4G0K	Green et al., 2012
GHR		*C. glutamicum*	–	1,2-Ethanediol; Glycerol	3M1G	Unpublished
GHR		*P. chrysosporium*	GSH	–	3PPU	Meux et al., 2011
GHR	PcpF	*S. chlorophenolicum*	–	–	4FQU	Green et al., 2012
GHR		*G. bronchialis*	–	–	4PTS	Unpublished
GSTL	GSTL3	*P. trichocarpa*	GSH	Ca^{2+}	4PQI	Lallement et al., 2014
GSTL	GSTL1	*P. trichocarpa*	GSH	Na^+	4PQH	Lallement et al., 2014

Available Cys-GST structures have been retrieved from the RCSB Protein data bank (http://www.rcsb.org/pdb/home/home.do). Only the first solved structures of wild-type isoforms have been listed, but for several proteins as human CLIC or GSTOs or E. coli YqjG, other structures have been obtained either for mutated proteins or for wild-type proteins in complex with GSH or another second ligand. For instance, in the case of E. coli YqjG, there are 3 structures described in the same study, one in apoform, one with GSH and one with GS-menadione. Beyond GSH, structures of Cys-GSTs with physiological substrates have been obtained in rare cases. The compounds indicated in the ligand 2 column essentially come from crystallization solutions.

**GSTO3-3 from B. mori is phylogenetically related to and classified as GSTOs although it displays an asparagine instead of the catalytic cysteine. GTS is for glutathione sulfonate.*

organisms, PcGHR1/Xi GST from *P. chrysosporium*, YqjG from *E. coli*, and its ortholog from *Corynebacterium glutamicum*, *Gordonia bronchialis*, and *Sphingobium chlorophenolicum* namely PcpF, but none from plants (Meux et al., 2011; Green et al., 2012).

ENZYMATIC PROPERTIES AND PHYSIOLOGICAL ROLES

As already mentioned, owing to the presence of a catalytic cysteine residue, Cys-GSTs have particular enzymatic properties since they should in principle catalyze deglutathionylation reactions by performing nucleophilic attacks on various GSH-conjugated substrates (Board et al., 2000; Dixon et al., 2002; Meux et al., 2011). Accordingly, most if not all GSTLs, GHRs, GSTOs and DHARs characterized so far exhibit thiol-transferase and DHAR activities but no transferase, peroxidase or isomerase activities except for a Beta GST from *Proteus mirabilis*, which possesses a slight peroxidase activity on cumene hydroperoxide (k_{cat} around 0.01 s^{-1}) and a non-negligible GSH transferase activity on CDNB (k_{cat} around 2 s^{-1}) (**Table 5**) (Federici et al., 2010). This was surprising since the transferase, peroxidase or isomerase activities are usually specific to Ser- or Tyr-containing GSTs as Phi, Tau, and Zeta GSTs. Indeed, it necessitates the activation of thiolate form of glutathione for direct glutathionylation reaction toward non-conjugated substrates (Dirr et al., 1994; Armstrong, 1997; Roxas et al., 1997). In the absence of known physiological substrates, hydroxyl-ethyl disulfide (HED), and DHA are often used to characterize the activity of recombinant GSTs as well as glutaredoxins, but it turns out that most glutathione-dependent oxidoreductases display such activities with very similar kinetic parameters (**Table 5**). The only notable exception is DHARs, for which DHA reduction is truly relevant. Consistently, they reduce DHA into ascorbate with a better efficiency (k_{cat} around 10^4 s^{-1}, k_{cat}/K_m around 10^7 $M^{-1}.s^{-1}$) compared to the other enzymes (k_{cat} around 10^2 s^{-1}) and to the reduction of glutathionylated-mercaptoethanol, the product formed upon incubation between GSH and HED (k_{cat} around 10^2 s^{-1}) (**Table 5**) (Dixon et al., 2002). The DHAR-mediated DHA

Table 5 | Enzymatic and ligandin activities detected for Cys-GSTs.

		GSTL	DHAR	GHR	mPGES-2	GSTB	GSTO
Thiol-transferase		10–10^{2} [abc]	10–10^{2} [a]	10^{2}–10^{3} [e]	?	?	10^{2}–10^{3} [egm]
DHA reductase		10–10^{2} [abc]	10^{3}–10^{4} [abd]	10–10^{2} [e]	?	?	10–10^{3} [egm]
Glutathionylation		nd [ac]	nd [a]	nd [e]	?	10–10^{3} [ij]	nd–1 [m]
Deglutathionylation	PAP-SG	10^{2}–10^{3} [c]	?	nd [e]	?	?	10^{3}–10^{4} *** [gmn]
	(Cl)Qui-SG	nd [c]	?	10^{2}–10^{3} [ef]	?	?	nd [g]
	TET-SG	nd [c]	?	?	?	?	10^{3} [g]
	Q-SG	1–10^{2} [bc]	?	?	?	?	?
PGH_2 isomerization		?	?	?	10^{2} * [h]	?	?
PGH_2 degradation		?	?	?	10^{3} ** [h]	?	?
Peroxidase		nd [ac]	nd [a]	nd [e]	?	0.1 [k]	nd [m]
Esterase		0.01–0.1 [c]	?	nd [g]	?	?	10 **** [g]
Ligandin		?	?	?		Antibiotics [kl]	Nitro-phenacyl glutathione [o] Tocopherol esters [p]

The data representing turnover numbers in min^{-1} have been extracted from the following references: [a] (Dixon et al., 2002); [b] (Dixon and Edwards, 2010b); [c] (Lallement et al., 2014); [d] (Tang and Yang, 2013); [e] (Meux et al., 2011); [f] (Lam et al., 2012); [g] (Meux et al., 2013); [h] (Yamada and Takusagawa, 2007); [i] (Allocati et al., 2000); [j] (Allocati et al., 2008); [k] (Perito et al., 1996); [l] (Allocati et al., 2009); [m] (Board et al., 2000); [n] (Board and Anders, 2007); [o] (Brock et al., 2013); [p] (Sampayo-Reyes and Zakharyan, 2006).

*Nd, not detected; ? not examined; *GSH-independent activity, **GSH-dependent activity, ***a slightly different substrate, acetophenone, was used (Board and Anders, 2007), **** k_{cat}/K_m in $mM^{-1}.min^{-1}$.*

reduction follows a ping-pong mechanism (Dixon et al., 2002; Shimaoka et al., 2003).

Based on several previous studies, a proposed catalytic mechanism that should apply for any glutathionylated substrate and any Cys-GST is presented in **Figure 6**. Since many Cys-GSTs characterized so far either structurally or biochemically have been shown to form mixed disulfides with GSH, there is little doubt that the catalytic cysteine performs a nucleophilic attack on GSH-conjugated substrates. The catalytic cysteine of Cys-GSTs becomes glutathionylated while the product of the reaction is released. The regeneration of these glutathionylated GST forms requires a GSH molecule, forming GSSG as another end product. While reduced Cys-GSTs are ready for another catalytic cycle, GSSG will be reduced back to GSH by glutathione reductase at the expense of NADPH. Since most Cys-GSTs have a single cysteine in the active site motif, they should follow this reaction mechanism. However, a few isoforms have an additional cysteine in the active site. This is the case of some DHAR isoforms which have CPFC active sites. For instance, *A. thaliana* DHAR3 was shown to form an intramolecular disulfide upon GSSG treatment by mass spectrometry (Dixon et al., 2002). Hence, it is possible that it constitutes either an intermediate step of the catalytic mechanism or possibly in other circumstances a protective mechanism that prevents over-oxidation of the catalytic cysteine into sulfenic, sulfinic, or sulfonic acid forms. Whatever the explanation is, the reduction of this disulfide would require a dithiol-disulfide exchange reaction. The most likely possibility is that it involves the successive intervention of two glutathione molecules, but another possibility is that a thioredoxin participates to this reduction step. Indeed, *A. thaliana* DHAR3 was isolated at least in two previous studies aiming at identifying thioredoxin targets (Marchand et al., 2004, 2006).

Besides glutathionylated-mercaptoethanol which contains a sulfur-sulfur bond, other glutathionylated substrates used so far have carbon-sulfur bonds (Meux et al., 2011, 2013; Lam et al., 2012; Lallement et al., 2014). For instance, beyond their DHAR activity, fungal and bacterial GHRs characterized so far efficiently reduce glutathionylated (chlorinated) (hydro)quinones with k_{cat} around 10^{3} s^{-1} and k_{cat}/K_m up to 10^{6} $M^{-1}.s^{-1}$ (**Table 5**) (Huang et al., 2008; Xun et al., 2010; Lam et al., 2012). However, there are some contrasting data in the literature. Some GHRs seem unable to catalyze the deglutathionylation of GSH conjugated-oxidized quinones and would be specific of glutathionylated reduced forms (Lam et al., 2012). On the other hand, using menadione as a substrate, a fungal GHR proved to deglutathionylate both forms with similar rates, but it is more efficient with the reduced forms because of a much better affinity (Meux et al., 2011). The latter observation points to the importance of the alcohol function for GHR recognition. Despite the above-mentioned discrepancy, it appears that GHRs are central to the regulation of the quinone redox state, likely preventing toxicity of quinones, either naturally present or found as environmental pollutants. Indeed, benzoquinones can covalently react with diverse macromolecules whereas hydroquinones, conjugated or not with glutathione, are prone to auto-oxidation forming reactive oxygen species. Since the major quinone forms found in the cells, ubiquinone and plastoquinone, are located into membranes and do not have electrophilic carbon groups that could be substituted by GSH, the question of the GHR physiological substrates is still open. Several other compounds often derived from lipids or fatty acids have alcohol functions and reactive electrophilic groups that might constitute possible substrates. As explained below, strategies aiming at identifying physiological substrates/ligands have been recently developed for other GSTs and they should be applied to GHRs. It is also possible that GHRs have protein substrates. For instance, it was proposed that the role of *S. cerevisiae* GTO1 could be related to the redox regulation of a Str3 cystathionine beta-lyase (Barreto et al., 2006). To date, the activity assays clearly separate GHRs from GSTLs and GSTOs which often catalyze the reduction of

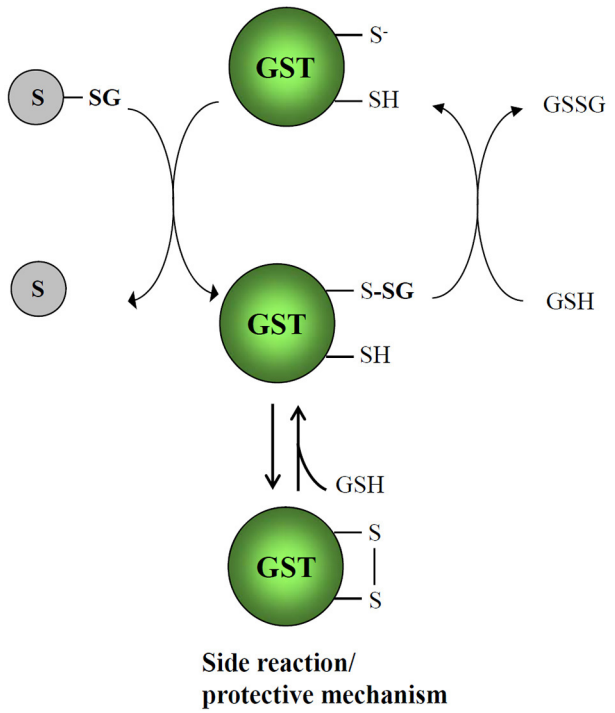

FIGURE 6 | Catalytic mechanisms of Cys-GSTs. The deglutathionylation of GSH-conjugated substrates occurs via the nucleophilic attack of the catalytic cysteine which is assumed to be at least partially under the thiolate form at physiological pH, owing to a decreased pKa value. Consequently, the catalytic cysteine is itself glutathionylated and it is regenerated using a glutathione molecule. For Cys-GSTs having another cysteine either in the active site (some DHAR isoforms) or at proximity (some GSTL isoforms), the identification of proteins with an intramolecular disulfide suggests that this might constitute either an intermediate step of the catalytic mechanism or more likely a protective mechanism that prevents oxidation of the catalytic cysteine into sulfenic acid forms or eventually higher oxidized forms as sulfinic or sulfonic acid forms. In the case of the formation of a disulfide an additional glutathione molecule would be required. It may be that thioredoxin participate to this reduction step as DHAR was isolated as a thioredoxin targets.

the same glutathionyl derivatives. GSTLs and GSTOs do not catalyze the deglutathionylation of glutathionylated quinones (Meux et al., 2013; Lallement et al., 2014). However, contrary to GHRs, GSTOs and GSTLs perform deglutathionylation of glutathionyl tetralone and/or acetophenone-derivatives with relatively good catalytic constants (k_{cat} around 10^4 s^{-1}) (**Table 5**) (Meux et al., 2013; Lallement et al., 2014) and they exhibit a weak esterase activity on the fluorescent probe chloromethyl fluorescein diacetate (CMFDA) (Meux et al., 2013; Lallement et al., 2014). This probe was initially used to identify tetralone as a GSTO substrate by competition experiments. Incidentally, one of the reported difference between GSTLs and GSTOs is that only GSTOs have the ability to remove the bound GSH molecule on glutathionyl tetralone (Meux et al., 2013; Lallement et al., 2014).

One of the major current challenge concerning Cys-GSTs and other GSTs is to identify relevant physiological substrates. One possibility to achieve this goal is to screen chemical libraries or cellular extracts by competition assays using fluorescent probes such as CFMDA or 8-anilino-1-naphthalenesulfonic acid (ANS). This has been successful in several cases both for Cys-GSTs (Son et al., 2010; Meux et al., 2013) and for those having a catalytic serine (Mathieu et al., 2012, 2013). Besides, Dixon and co-workers have identified several flavonoids derived from kaempferol which can bind tightly to GSTLs from Arabidopsis and wheat by ligand fishing approaches (Dixon and Edwards, 2010a). These approaches consist in isolating by affinity chromatographies and identifying natural physiological substrates from plants using *in vitro* and *in vivo* approaches. Both methods rely on the use of tagged proteins either by mixing them with crude or fractionated extracts or to secondary metabolite enriched-extracts, or by expressing them *in planta* in order to really trap physiological protein-substrate complexes. They proved to work also with Phi and Tau GSTs, the latter binding porphyrin intermediates and fatty acids (Dixon et al., 2008, 2011; Dixon and Edwards, 2009). It was then confirmed by enzymatic analyses that GSTLs from Arabidopsis, wheat and poplar can perform deglutathionylation of glutathionylated quercetin (Dixon and Edwards, 2010b; Lallement et al., 2014). However, the fact that the turnover numbers are quite low and similar to those obtained with other oxidoreductases (Grxs, Trxs, GSTOs, GHRs, and DHARs) from various organisms (Lallement et al., 2014) and that a quercetin derivative was also isolated from a ligand fishing experiment performed with a Phi GST (Dixon et al., 2011) raises the question of a specific role of GSTLs in quercetin recycling and in the maintenance of a reduced flavonoids pool. Overall, this may indicate that flavonoids are GST substrates, but it does not tell exactly which enzyme(s) is (are) really efficient *in vivo*.

From a biochemical point of view, mPGES-2 can hardly be compared with other Cys-GSTs. Indeed, although it has been shown that they do not display GSH transferase activity, none of the usual activities of Cys-GSTs were assayed (Watanabe et al., 1999). Since mammalian mPGES-2s have a defined role in prostanoid metabolism, all studies primarily investigated the PGH_2 conversion into PGE_2 (Watanabe et al., 1997). However, an issue was the observation that mPGES-2 activity was partially independent from glutathione and that DTT induced a 4-fold better efficiency of the proteins (Tanikawa et al., 2002). A recently solved structure of a heme-bound mPGES-2 allowed solving this discrepancy. Indeed, it seems that the isomerization activity is catalyzed by a heme-free enzyme, whereas heme-bound mPGES-2s can degrade PGH_2 into hydroxyl heptadecatrienoic acid and malondialdehyde, instead of converting it to PGE_2 (Jania et al., 2009; Takusagawa, 2013). This activity relies to the binding of a heme, which is stabilized by hydrogen bonds when a glutathione is present in the active site (Yamada and Takusagawa, 2007; Takusagawa, 2013). Hence, this may help explaining that the activity of isomerization is increased by adding DTT as it contributed to remove both GSH and heme from the active site. Overall, the current view in animals is that the physiological role of mPGES-2s is related to the degradation of PGH_2 rather than to its isomerization into PGE_2. This is also consistent with the fact that mPGES-1s also catalyze the GSH-dependent PGH_2 isomerization into PGE_2 with good efficiencies (Thoren et al., 2003). Since there is no PGH_2 and PGE_2 in plants, the physiological roles and substrates of mPGES-2 are unclear. Looking for

possible related candidate molecules in plants, oxylipins might constitute such substrates. Indeed, these molecules, derived from the enzymatic and non-enzymatic peroxidation of fatty acids, exhibit a reactive carbonyl structure, which makes them highly reactive electrophilic species and they are formed at proximity of mPGES-2 localization (Farmer and Mueller, 2013). These compounds participate to numerous developmental processes and to stress response. It is for instance documented that the expression of some GSTs is induced by 12-oxo-phytodienoic acid (OPDA), a phytohormone precursor, and phytoprostane A1 (PPA_1) (Mueller et al., 2008). Consequently, it was hypothesized that GSTs might reduce the reactive cyclopentenone ring to an unreactive ring. Hence, by glutathionylating or deglutathionylating these molecules, GSTs and possibly mPGES-2s could modulate the concerned signaling pathways. To conclude, although Cys-GSTs are encoded by multigenic families, there is a pressing need to perform reverse genetics by systematically generating single or multiple mutant lines or overexpressing lines to delineate the exact function of these proteins.

MATERIALS AND METHODS

SEQUENCE RETRIEVAL, STRUCTURAL ALIGNMENT AND PHYLOGENETIC ANALYSES

Sequences have been retrieved by iterative blastp analyses using a set of as variable GST sequences as possible either from the cyanobase (http://genome.microbedb.jp/cyanobase/) for cyanobacteria, from the jgi genome portal (http://genome.jgi.doe.gov/) for most algae and from the version 10 of the phytozome portal (http://phytozome.jgi.doe.gov/pz/portal.html) for terrestrial plants. When needed and possible, sequences have been completed and validated by analyzing the presence of ESTs using tblastn analyses against the NCBI protein databank. Sequences were then aligned with PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php) (Pei et al., 2008) and alignment manually adjusted with Seaview software (Gouy et al., 2010). The phylogenetic tree was constructed with BioNJ (Gascuel, 1997) in Seaview and edited with Figtree software (http://tree.bio.ed.ac.uk/software/figtree/).

SUBCELLULAR LOCALIZATION

The GST subcellular localization was defined based on the available literature, as well as from database mining using TAIR v10, and from the following prediction softwares, Predotar, TargetP, and Wolfpsort.

EXPRESSION ANALYSES

For developmental conditions, expression data from Gene Expression Map of Arabidopsis Development (Schmid et al., 2005) were retrieved using the eFP browser (Winter et al., 2007). Each gene was normalized to its maximum expression within the selected dataset. Further, the number of developmental conditions was reduced to eight classes in order to gain a better overview of the overall expression profile of each GST during Arabidopsis development. These classes were grouped together and their relative expression averaged as follows: Mature seeds (stages 8, 9, and 10 without siliques and dry seed), Developing seeds (stages 3, 4, and 5 with siliques), Pollen (mature), Flowers (stages 9, 10/11, 12, and 15), Senescing leaves, Leaves (rosette leaves 4, 6, 8, and 10), Shoot Apices (vegetative, transition, and inflorescence), and Roots (from seedlings and mature rosettes).

For perturbation conditions, expression data for each GST were obtained from Genevestigator V3 (Hruz et al., 2008). Only data with a p-value < 0.001 were included in the analysis. Perturbations in the resulting lists were grouped into 11 classes: Biotic stress (Bs), Chemicals (Che), Germination (Ge), Light (Li), N-starvation (-N), Fe-deficiency (-Fe), Salt stress (Sa), Hypoxia (Hy), Drought (Dr), Heat (He), and Cold (Co).

Data were analyzed using Open Office Calc (Apache), graphed using PRISM (GraphPad) and clustered using Multi-experiment Viewer (MeV).

AUTHOR CONTRIBUTIONS

Pierre-Alexandre Lallement, Arnaud Hecker, and Nicolas Rouhier carried out the *in silico* genome analyses. Bastiaan Brouwer and Olivier Keech performed the transcriptome and subcellular prediction analyses. All authors participated to the writing of the manuscript, have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphar.2014.00192/abstract

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Maintenance of glutathione levels and its importance in epigenetic regulation

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Glutathione (GSH) is present in almost all cell types playing an important function in organisms. It is the main antioxidant in many cell types and it also regulates the function of proteins, including transcription factors (reviewed in Pallardó et al., 2009; Markovic et al., 2010; García-Giménez et al., 2013a).

Over recent years, growing evidence has suggested a link between GSH metabolism and the control of epigenetic mechanisms. Epigenetics is defined as the mitotically/meiotically heritable changes in gene expression that are not due to changes in the primary DNA sequence. This link between GSH and epigenetics occurs at different levels. Hence, GSH can affect DNA and histone methylation, and also it binds to histones throughout the cysteines in the histone H3. Pioneer work showed how oxidized GSH inhibits the activity of S-adenosyl methionine synthetase, MAT1A. This key enzyme is involved in the synthesis of S-adenosyl methionine (SAM), which is used by DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) as a substrate for DNA and histone methylation, respectively (Pajares et al., 1992; Martinez-Chantar and Pajares, 1996). Therefore, it is possible to alter the methylation status of the genome and modify the epigenetic signature in cells by modulating SAM levels. Methylation of DNA and histone constitutes one of the most studied chemical modifications in the epigenetic code, which shapes gene-expression patterns usually, but not always, repressing gene transcription. Interestingly, replenishment of GSH levels recovers the activity of MAT1A (Mato et al., 2002) thereby contributing to the homeostasis of DNA and histone methylation.

GSH influence on the epigenetic mechanisms may go beyond mere regulation of SAM levels by the mechanism described above. Our recent results (García-Giménez et al., 2013b) report the implication of GSH in the regulation of epigenetic mechanisms by redox phenomena. We recently described the S-glutathionylation of histone H3 as a new post-translational modification, PTM in the histone code. In this modification, GSH binds to Cys110 in histone H3 (Histone H3 variants, H3.1, H3.2, and H3.3) producing changes in the stability of the nucleosomes and altering the chromatin structure by decreasing the proportion of α-helices. Interestingly, we also observed how S-glutathionylation of H3 increased in proliferating cells but not in quiescent cells, suggesting that GSH modifies the structure of the chromatin during cell proliferation. The implications of these results are relevant because the opening of the chromatin by histone glutathionylation contributes to the binding of the replication machinery to the DNA. Furthermore, the ability of GSH to open the chromatin may increase the susceptibility of DNA to the attack of DNA-interacting drugs. Our results agree with those obtained by de Luca et al. in which glutathionylation of H3 may facilitate the interaction of doxorubicin with DNA, thereby increasing the effect of the treatment to doxorubicin-resistant MCF7 breast cancer cells (de Luca et al., 2011).

However, the epigenetic mechanisms described above are just two examples of the many epigenetic events in which GSH may be involved. For example, oxidative stress induces the formation of methionine sulfoxide from methionine and this molecule can immediately react with radical hydroxyl to generate a methyl radical that non-enzymatically and non-specifically methylates cytosine in the DNA (Kawai et al., 2010). This phenomenon could produce deleterious effects in our epigenome (Lewandowska and Bartoszek, 2011). Fortunately, a GSH-dependent enzymatic mechanism that prevents the production of methionine sulfoxide does exist. The GSH/glutaredoxin system can regenerate the activated form of methionine sulfoxide reductase, MSR, the enzyme that converts methionine sulfoxide to methionine (Kim, 2012). Therefore, GSH/glutaredoxin/MSR prevents the generation of the methyl radical and contributes to the regeneration of methionine, which in turn is introduced in the methionine cycle to recover the SAM levels.

Furthermore, it was recently reported that oxidative stress affects methionine synthase (MS), an important enzyme in the regeneration of methionine from homocysteine (Muratore et al., 2013). A decrease in MS activity results in elevated levels of homocysteine, which is associated with blindness, neurological symptoms, and birth defects including encephalopathy and megaloblastic anemia (Outteryck et al., 2012). For this reason, cellular redox control in which GSH, as the main cellular antioxidant is involved, confers GSH the relevant role of controlling key enzymatic processes. These are directly

related to the homeostasis of DNA methylation to prevent extensive DNA and histone methylation changes, which in turn would produce harmful consequences.

A number of diseases have been described in which impaired methylation and changes in GSH levels have been reported. This is the case of several psychiatric disorders, like schizophrenia (Regland et al., 1994; Yao et al., 2006), bipolar disorder (Frey et al., 2007; Kuratomi et al., 2008), and autism (James et al., 2004, 2006) which underscores the role of GSH and DNA methylation in these diseases.

Therefore, the evidence reported in this manuscript indicates that pharmacological interventions designed to recover GSH homeostasis favor the correct function of the methionine cycle (MAT1A and MS), the replenishment of SAM, and prevent the non-specific methylation reactions by reducing MSR. Furthermore, as mentioned above, oxidative stress controls the activity of MS, which produces methionine. This amino acid is then used by the MAT1A (which is also controlled by the GSH/GSSG ratio) to produce SAM. This intermediate, besides participating as a substrate for DNMTs and HMTs, is also one of the precursors of the transulfuration pathway that finally produces cysteine, which is used for the synthesis of GSH.

Consequently, efforts to increase cellular GSH concentration by direct administration of GSH analogs have been a challenge over recent decades (reviewed in Wu and Batist, 2013). Some approaches were developed for clinical application in order to increase the stability and uptake of GSH by cells (Sheh et al., 1990; Shibata et al., 1995). Therefore, by controlling the levels of both metabolites we can counterbalance the cellular redox and methylation status. In so doing, it was found that the administration of drugs (i.e., butanedisulphonate) that increase the levels of SAM produces the recovery of GSH levels. This strategy was proposed as a therapeutic approach in HIV patients with central nervous system affectation who presented decreased concentrations of SAM and GSH (Castagna et al., 1995). However, the recovery of GSH concentration in human tissues is difficult due to its biochemical and pharmacokinetic properties but different strategies are being developed to replenish GSH levels (for a review see Cacciatore et al., 2010). Due to the short half-life of GSH in human plasma and its difficulty to penetrate the plasma membrane, it is necessary to provide a high dose to recover physiological levels of the GSH. The use of prodrugs is the most promising approach (Anderson and Luo, 1998) and they present the possibility to be used as modulators of GSH and SAM levels in this kind of diseases.

Another possible approach is the use of co-drugs. Two different compounds are administered simultaneously, when using this class of chemicals. Both compounds are linked to each other by using a covalent bond that is cleavable which regenerates the original drug with adequate bioavailability. Investigations in this type of compounds have already been reported (Ehrlich et al., 2007). In addition, as we have described, oxidative stress produces methionine sulfoxide, which is epigenetically toxic. Therefore, the use of antioxidants could prevent the formation of this oxidized amino acid. It was recently reported that antioxidants like vitamin C regulate epigenetic mechanisms. It has been described how vitamin C, modulates the activity of Tet methylcytosine dioxygenases, which are involved in the formation of 5-hydroxymethylcytosine, thus participating in the demethylation of DNA (Chen et al., 2013; Minor et al., 2013). These authors described how vitamin C by acting as a cofactor for Tet proteins enhances the 5-hidroxymethylation of cytosine (5-hmC) at critical loci for somatic cell reprogramming (Chen et al., 2013; Minor et al., 2013). Thus, they described for first time the role of ascorbate modulating the epigenetic control of genome. The authors also studied the effect of GSH as reducing agent, but they observed that levels of 5-hmC were not affected (Minor et al., 2013). However, in their experiments the authors used GSH, which do not penetrate the plasma membrane. It would be very relevant to reproduce their experiments by incubating MEFs with glutathione ethyl ester, which is able to cross the cellular membranes to conclude if GSH can affect the levels of 5-hmC in the DNA.

The related mechanisms described in this manuscript place the GSH and redox control in the landscape of the epigenetic regulation. The modulation of GSH and SAM levels has the potential to control oxidative stress and epigenetics. Therefore, the design of strategies and the synthesis of therapeutic drugs that help to reestablish the levels of these key molecules are crucial for therapeutic interventions in epigenetic-related diseases.

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Regulation of epigenetic traits of the glutathione *S*-transferase P1 gene: from detoxification toward cancer prevention and diagnosis

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Glutathione *S*-transferases (GSTs) are phase II drug detoxifying enzymes that play an essential role in the maintenance of cell integrity and protection against DNA damage by catalyzing the conjugation of glutathione to a wide variety of exo- and endogenous electrophilic substrates. Glutathione *S*-transferase P1 (GSTP1), the gene encoding the pi-class GST, is frequently inactivated by acquired somatic CpG island promoter hypermethylation in multiple cancer subtypes including prostate, breast, liver, and blood cancers. Epigenetically mediated GSTP1 silencing is associated with enhanced cancer susceptibility by decreasing its "caretaker" gene function, which tends to promote neoplastic transformation allowing cells to acquire additional alterations. Thus, this epigenetic alteration is now considered as a cancer biomarker but could as well play a driving role in multistep cancer development, especially well documented in prostate cancer development. The present review discusses applications of epigenetic alterations affecting GSTP1 in cancer medicine used alone or in combination with other biomarkers for cancer detection and diagnosis as well as for future targeted preventive and therapeutic interventions including by dietary agents.

Keywords: GSTP1, cancer, epigenetics, DNA methylation, histone modifications, epimutations, biomarker

INTRODUCTION

As one of the driving forces behind the cellular detoxification machinery, glutathione *S*-transferases (GSTs) and especially the pi class glutathione *S*-transferase P1 (GSTP1) is currently in the focus of the cancer research community, evaluating the relevance of GSTP1 epimutations for cancer development and its potential as a major epigenetic cancer biomarker.

The human GST multi-gene superfamily is encoding for various ubiquitous cytosolic or soluble, mitochondrial and microsomal as well as peroxisomal homo- and heterodimeric transferases (Di Pietro et al., 2010). Despite the multifunctionality of these proteins, GSTs are best known for their ability to transfer the tripeptide gamma-glutamyl-cysteinyl-glycine, also known as glutathione (GSH) to a wide variety of highly genotoxic and cell-damaging molecules, either directly occurred from the extracellular environment or from the intracellular detoxification metabolism. In most albeit not all cases, glutathione *S*-conjugation generates a less or non-toxic product with improved water solubility, favoring the exportation out of the cell and thereby contributing to DNA damage prevention and protection of the cellular integrity (Baden et al., 2011). Moreover, several GST isozymes are implicated in cell signaling, interfering for example with the MAPK signaling cascade, which is involved in the regulation of cell cycle, proliferation and cell death (Wang et al., 2001; Laborde, 2010).

Regardless the importance of GST activity for cellular vitality and health, the GST gene cluster is a hotspot for DNA sequence mutations that leads to the expression of active but functionally different GST variant proteins. Accordingly, cells expressing less active GST isoforms are more sensible to GST-metabolized toxins compared to cells with balanced GST activity. In a worst case-scenario, cells are incapable to degrade carcinogens or stress-induced toxic intermediates, thus increasing their susceptibility to undergo further steps toward cancer progression or event other diseases (Deep et al., 2012). The GSTP1*B (Val105) allele is often mentioned within the context of genetic polymorphisms, a GSTP1 variation which is characterized by an A→G sequence transition in codon 105 of exon 5, leading to the exchange of isoleucine by valine, thus decreasing its catalytic activity associated with reduced cell detoxification ability (Saxena et al., 2012).

IMPORTANCE OF GLUTATHIONE *S*-TRANSFERASE P1 CLASS IN CANCER DEVELOPMENT

Furthermore, the previously mentioned cytosolic GSTP1 isoenzyme consist of one of the best-studied variants of the GST metabolism. Located on chromosome 11, the GSTP1 coding region is controlled by a large CpG island (CGI) upstream of the transcription start site in the promoter region. Both areas are separated by a long ATAAA repetitive stretch, which probably acts as an insulator to separate different epigenetic states such as methylation of the CGIs (Millar et al., 2000). Moreover, various transcription factors such as specificity protein 1 (SP1), activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and GATA1 were reported to play an important role in the regulation of GSTP1 expression (**Figure 1**; Moffat et al., 1996; Duvoix et al., 2003b, 2004b; Schnekenburger et al., 2003; Morceau et al., 2004).

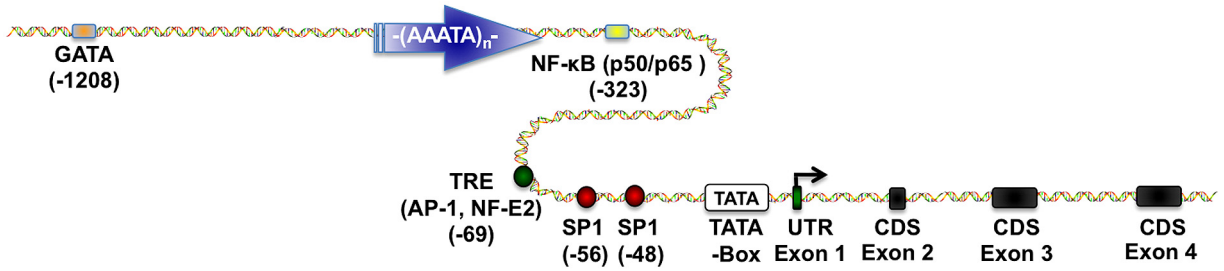

FIGURE 1 | Glutathione *S*-transferase P1 (GSTP1) regulatory elements. This scheme depicts essential transcriptional regulatory elements known to regulate GSTP1 gene expression. Proximal promoter region contains (i) two SP1 sites (Morrow et al., 1989), (ii) one TPA-response element (TRE) binding (activator protein) AP-1 and nuclear factor, erythroid 2 (NF-E2) depending on the cellular environment (Borde-Chiche et al., 2001b; Duvoix et al., 2003a,b, 2004a,b), (iii) one nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding site (Morceau et al., 2004), and (iv) a GATA-1 binding site (Schnekenburger et al., 2003, 2004). Figure was generated by using ScienceSlides.

Glutathione *S*-transferase P1 is also involved in cell death regulation, interacting with apoptotic signaling pathways as for example c-Jun NH2-terminal kinase (JNK1), ERK1/ERK2, or tumor necrosis factor receptor-associated factor 2 (TRAF2; Ruscoe et al., 2001; Wu et al., 2006). In correlation to its biotransformation and detoxification ability, GSTP1 is expressed in most cells and particularly in those that are in contact with the external environment such as cells of the urinary, digestive, and respiratory tract (Terrier et al., 1990; Yuan et al., 2008).

Increased levels of GSTP1 expression can also be indicative for enhanced detoxification activity due to xeno- or endo-biotic exposure implicating oxidative stress (Kanwal et al., 2014). Accordingly, increased GSTP1 expression is often detected in many cancers (e.g., breast, colon, stomach, pancreas, bladder, lung, head and neck, ovary and cervix, soft tissue sarcoma, testicular embryonic carcinoma, meningioma, and glioma), which is associated to enhanced detoxification activity, thus protecting cancer cells against cytotoxic and cytostatic drugs (Ruzza et al., 2009). In contrast, knockout experiences in mice showed that loss of GSTP1 expression leads to increased cancer susceptibility (Henderson et al., 1998; Ketterer, 1998).

REGULATION OF CELLULAR ACTIVITIES THROUGH EPIGENETIC MECHANISMS

Alterations in the epigenetic setup are as important as genetic aberrations and may cooperate in cancer genesis. While classical gene mutations are region-limited, epimutations often occur early in cancer development and have a genome-wide impact, boosting on the one hand the expression of cell survival genes or proto-oncogenes and deactivating on the other hand tumor suppressor genes (TSGs), DNA repair mechanisms as well as cell division brakes, thus leading to carcinogenesis (Esteller, 2008; Florean et al., 2011; Karius et al., 2012; Schnekenburger and Diederich, 2012; Seidel et al., 2012; Blancafort et al., 2013).

In addition to the genetic information encoded by the primary DNA sequence, epigenetic mechanisms add a layer of regulation of the information and includes DNA methylation, histone modifications as well as regulation by non-coding RNAs (Esteller, 2008; Florean et al., 2011; Karius et al., 2012; Schnekenburger and Diederich, 2012; Seidel et al., 2012; Blancafort et al., 2013). This interacting cluster of epigenetic regulators provides an epigenetic memory, transferring epigenetic information through mitotic and meiotic cell divisions (Migicovsky and Kovalchuk, 2011). These reversible modifications are playing essential roles in gene regulation, X-inactivation, imprinting, and silencing of parasitic DNA elements (Jaenisch and Bird, 2003).

PATHOLOGICAL ALTERATIONS OF GSTP1 METHYLATION PATTERNS

DNA methylation, the addition of a methyl-group to cytosines is the most common epigenetic modification, inducing the reorganization of the gene locus and thus regulating gene expression (Smith and Meissner, 2013). However, tumor cells typically possess an aberrant methylation pattern associated with altered gene expression profiles, showing locally restricted hypermethylation of individual promoter regions involved in the silencing of TSGs. At the same time, genome-wide loss of DNA methylation is observed in tumor cells compared to healthy cells inducing chromosomal instability, loss of imprinting as well as the previously described oncogene activation.

In its function as a cellular "caretaker", attenuation of GSTP1 expression or activity by either genetic (e.g., deletion or mutation) or epigenetic alterations may reduce cellular detoxification capacity (**Figure 2**). In fact, the use of GSTP1 knockout mice demonstrated that loss of GSTP1 expression increases sensitivity to metabolic or environmental toxins and promotes mutations and cancer development (Coughlin and Hall, 2002a,b).

Prostate cancer

Promoter hypermethylation leading to epigenetic silencing of GSTP1 gene expression is frequently detected in prostate cancer cells, the most commonly diagnosed type of malignancy among men in Western European countries and the second cause of cancer-related deaths among men worldwide (Brooks et al., 1998; Cairns et al., 2001; Jeronimo et al., 2002; Henrique and Jeronimo, 2004; Dumache et al., 2010; Ferlay et al., 2010). Interestingly, GSTP1 hypermethylation is strictly restricted to malignant cells including prostate cancer cells (PCa) as well as prostatic intraepithelial neoplasia (PIN). Detection of GSTP1 methylation in all types of body fluids of prostate cancer patients represents a promising epigenetic biomarker, which is already under evaluation for the application of new prognostic methods (Cairns

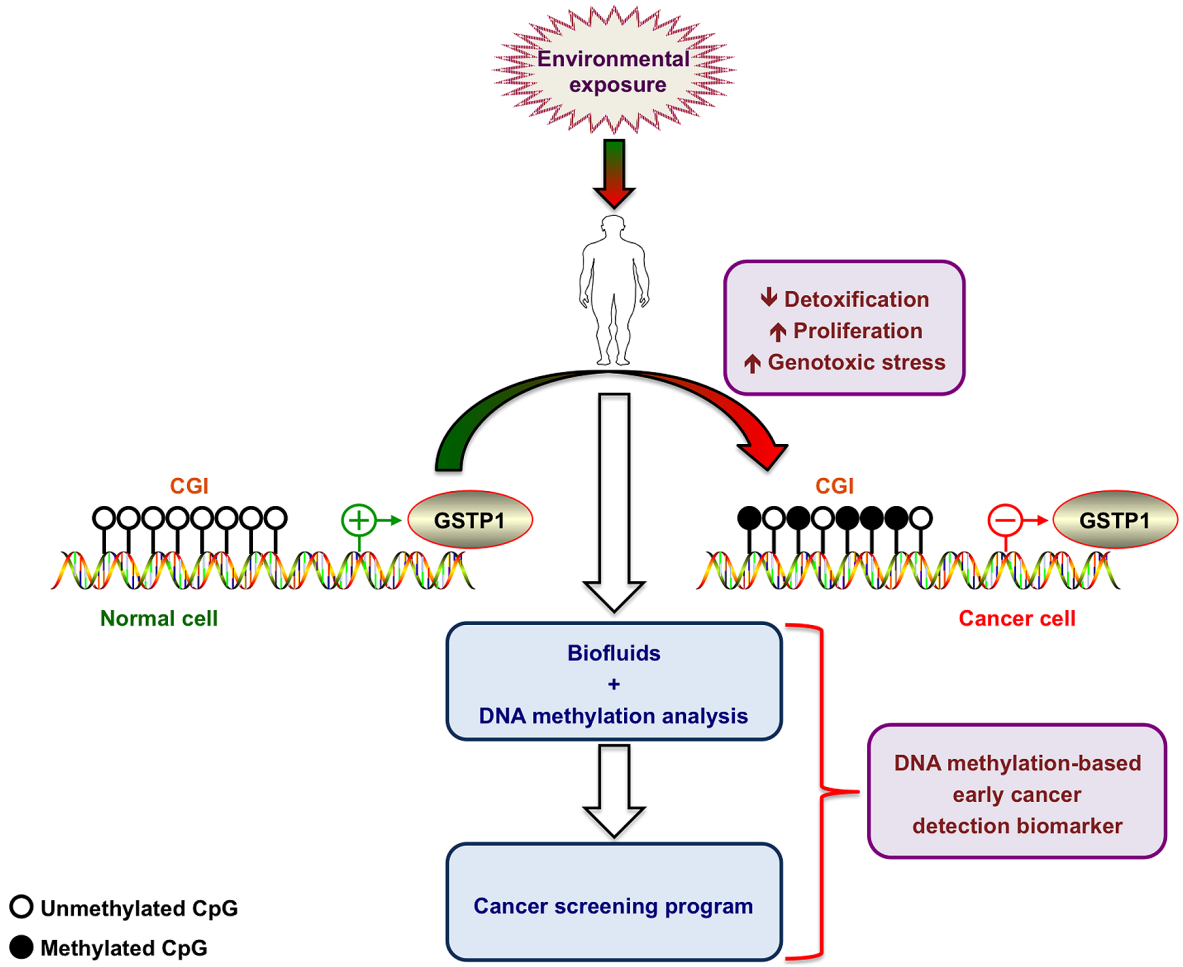

FIGURE 2 | Hypermethylation of the glutathione *S*-transferase P1 promoter as early cancer biomarker. Environmental exposure leads to progressive methylation of CpG islands (CGI) at the GSTP1 promoter region. The resulting decreased cell detoxification capacity leads to increased genotoxic stress and progressive accumulation of additional genetic alterations accompanied by an increased proliferation rate. DNA methylation analysis of the GSTP1 promoter in body fluids allows detecting progressive hypermethylation and turns this molecular feature into a valid early cancer biomarker. Figure was generated by using ScienceSlides.

et al., 2001; Esteller, 2008; Yang and Park, 2012; **Figure 2**). In contrast, GSTP1 promoter remains almost unmethylated in benign lesions, allowing distinguishing benign and cancerous transformations (Hopkins et al., 2007; Cao and Yao, 2010). Recently, Re et al. (2011) published that, beyond hypermethylation, chromatin remodeling by a combinatorial complex between estrogen receptor (ER) and endothelial nitric oxide synthase (eNOS) also represses transcription of prognostic genes that are down-regulated in PCa, such as GSTP1. In PCa cultured cells ER/eNOS causes GSTP1 repression by being recruited at estrogen responsive elements within its promoter favoring a local chromatin remodeling together with hypermethylated promoter sequences.

Lung cancer

Esteller et al. (1999) published that aberrant hypermethylation of the TSG p16, the putative metastasis suppressor gene death-associated protein kinase (DAPK), GSTP1, and the DNA repair gene O6-methylguanine-DNA-methyltransferase (MGMT) is observed in non-small cell lung cancer (NSCLC) tumors but not in any paired normal lung tissue. In primary tumors with methylation, 11 of 15 (73%) samples also had abnormal methylated DNA in the matched serum samples. More recently, Kim et al. (2005) concluded that DNA methylation status of CGI could be used as a predictor of long-term outcome for adenocarcinoma of the lung.

Leukemia and lymphoma

Borde-Chiche et al. (2001a) published that methylation of CpG sites of the basal GSTP1 promoter is an essential mechanism controlling GSTP1 gene expression in human leukemia. Karius et al. (2011) further investigated this mechanism and showed by bisulfite sequencing, methylation-specific PCR and combined bisulfite restriction analysis that the GSTP1 promoter was completely methylated in transcriptionally inactive in RAJI Burkitt's lymphoma and MEG-01 chronic myeloid leukemia cell

lines. In contrast, cell lines expressing GSTP1 exhibited an unmethylated and transcriptionally active promoter thus confirming a relationship between hypermethylation and repression of GSTP1 expression (Karius et al., 2011).

Rossi et al. (2004) investigated methylation of MGMT, DAPK, and GSTP1 and concluded that these alterations represent a major pathogenetic event in several B-cell malignancies. Inactivation of GSTP1 in gastric MALT lymphoma represents an additional mechanism favoring accumulation of reactive oxygen species to further promote lymphomagenesis. Finally, frequency of GSTP1 aberrant methylation in diffuse large B-cell lymphoma (DLBCL) also led to studies to validate the prognostic impact of such epigenetic alteration in these lymphomas. Nakamichi et al. (2007) established a correlation between promoter hypermethylation of GSTP1 and response to chemotherapy in DLBCL. According to the authors, the GSTP1 gene methylation status could be an indicator of drug response and a prognosticator for DLBCL (Nakamichi et al., 2007).

Breast cancer

Jhaveri and Morrow initially published that methylation status of GSTP1 promoter contributes significantly to the levels of GSTP1 expressed in ER-negative and ER+-positive breast cancer cell lines (Jhaveri and Morrow, 1998). Moreover, GSTP1 hypermethylation and therefore gene silencing was associated to increased grades of mammary phyllodes tumors. As such GSTP1 methylation patterns allow distinguishing two groups: one benign unmethylated group as well as samples presenting hypermethylated GSTP1 gene promoters in the borderline/malignant tumor group (Kim et al., 2009). Recently, Miyake et al. (2012) demonstrated that GSTP1 expression predicts poor pathological complete response to neoadjuvant chemotherapy in ER-negative breast cancer. Indeed, GSTP1 expression can predict pathological response to chemotherapeutic treatments with 5-fluorouracil/epirubicin/cyclophosphamide in ER-negative tumors but not in ER-positive tumors. Additionally, GSTP1 promoter hypermethylation might be implicated more importantly in the pathogenesis of luminal A, luminal B, and HER2-enriched tumors, than in basal-like tumors.

Liver cancer

According to Zhang et al. (2012) GSTP1 is transcriptionally silenced by promoter hypermethylation in several human cancer types including hepatocellular carcinoma (HCC). These results suggest that epigenetic inactivation of GSTP1 plays an important role in the development of HCC and exposure to environmental carcinogens may be related to altered methylation of genes involved in hepatocarcinogenesis (Zhang et al., 2012).

Other cancer subtypes

Similarly, the invasion potential of pituitary tumors and endometrial carcinomas was linked to reduction of GSTP1 expression and methylation frequency, indicating that epigenetically mediated down-regulation of GSTP1 expression may also contribute to aggressive pituitary tumor behavior (Chan et al., 2005; Yuan et al., 2008).

PATHOLOGICAL ALTERATIONS OF GSTP1 METHYLATION PATTERNS

Beyond DNA hypermethylation, which was the first epigenetic alteration to be discovered as an influencing factor for GSTP1 expression, histone modifications were discovered in the early 2000 s to play a regulating role in GSTP1 expression. Moreover, an interplay between histone reprogramming and DNA methylation was emerging. Bakker et al. (2002) initially showed that methyl-CpG binding domain protein (MBD)2 represses transcription from hypermethylated GSTP1 gene promoters in HCC cells connecting hypermethylation and reduced transactivation potential. Similarly, Lin and Nelson (2003) published that MDB2 mediates transcriptional repression associated with hypermethylated GSTP1 CGIs in MCF-7 breast cancer cells.

HATs (histone acetyltransferases) contribute to the regulation of gene expression, and loss or deregulation of these activities may link to tumorigenesis. Ohta et al. (2007) demonstrated that expression levels of HATs, p300, and CBP [CREB (cAMP-response-element-binding protein)-binding protein] were decreased during chemical hepatocarcinogenesis, whereas expression of MOZ (monocytic leukemia zinc-finger protein; MYST3), a member of the MYST [MOZ, Ybf2/Sas3, Sas2, and TIP60 (Tat-interacting protein, 60 kDa)] HAT family was induced. Exogenous MOZ induced GSTP1 expression in rat hepatoma H4IIE cells. These results suggest that during early hepatocarcinogenesis, aberrantly expressed MOZ may induce GSTP1 expression through the NF-E2-related factor 2 (Nrf2)-mediated pathway.

Okino et al. (2007) investigated chromatin changes on GSTP1 promoter associated with its inactivation in prostate cancer. Thus, treatment of LNCaP cells with the DNA demethylating agent 5-azacytidine also restored activating histone modifications on GSTP1 and as a result reactivated transcription. Authors concluded that, in the process of prostate carcinogenesis, activating histone modifications on GSTP1 are lost and subsequently DNA becomes methylated and inaccessible resulting in transcriptional silencing thus demonstrating interplay between histone reprogramming and promoter region methylation (Okino et al., 2007).

Karius et al. (2011) further contributed that histone marks and effector proteins associated with transcriptional activity could be detected by chromatin immunoprecipitation in GSTP1 expressing hypomethylated K-562 cell line. However, repressive chromatin marks and the recruitment of silencing protein complexes were found in the non-expressing hypermethylated RAJI and MEG-01 cell lines, again validating the interrelationship between DNA methylation and histone marks (Karius et al., 2011).

In prostate cancer cells, Hauptstock et al. (2011) used the histone deacetylase inhibitor depispeptide to reverse DNA hypermethylation and alter the histone modification pattern at GSTP1 promoter, including a reduction of H3K9me2/3 and H3K27me2/3 and an increase of H3K18Ac, thereby inducing GSTP1 mRNA re-expression. For these authors, successful therapy requires both, DNA demethylation and triggering activating histone modifications, to induce complete gene expression of epigenetically silenced genes and depsipeptide fulfills both criteria (Hauptstock et al., 2011).

SMALL REGULATORY RNA INVOLVED IN GSTP1 EXPRESSION

MicroRNAs (miRNAs) constitute a large family of regulatory non-coding (nc)RNAs (Morceau et al., 2013). These single stranded RNAs (17–25 nucleotides) are highly conserved during evolution and are generated by a multistage process. Their expression leads to post-transcriptional silencing of target genes by mRNA translation repression. MiRNAs target several mRNAs while a specific mRNA can be targeted by several miRNAs. Altogether these regulatory mechanisms also belong to epigenetic regulation.

Accumulating evidence suggest that, as most mRNAs, the level of GSTP1 transcripts can potentially be regulated by several miRNAs. Patron et al. (2012) published that miR-133b reduces GSTP1 expression by 2.1 fold in prostate cancer cells. In addition, miR-513a-3p sensitizes human A549 lung adenocarcinoma cells to chemotherapy by targeting GSTP1 (Zhang et al., 2012). Mutallip et al. (2011) showed that transient transfection of miR-133a repressed the expression of GSTP1 at mRNA and protein levels. Similar results were published in human bladder cancer (Uchida et al., 2013) and earlier in lung squamous cell carcinoma (Moriya et al., 2012).

Even though only limited information is available regarding the regulation of GSTP1 by miRNAs, we believe that further investigations could contribute to the development of new therapeutic miRNA-based anticancer strategies.

DIETARY REGULATORS OF GSTP1 EXPRESSION

Since progressive GSTP1 hypermethylation is a hallmark biomarker but potentially also a driver of prostate cancer progression, various research teams were looking for dietary intervention to lower the methylation burden of GSTP1 gene promoter. By re-expressing GSTP1, increased detoxification, and reduced levels of oxidative stress could potentially contribute to reduced prostate cancer progression and even to an abrogation on evolution toward invasive and metastatic disease.

Accordingly, many studies reported dietary nutrients or phytochemicals that present the potential to restore GSTP1 expression (Schnekenburger et al., 2014). For instance, Vardi et al. (2010) showed that soy phytoestrogens modify DNA methylation of GSTP1, RASSF1A, EPH2, and BRCA1 TSG promoters in prostate cancer cells. After treatment by phytoestrogens, demethylation of GSTP1, and EPHB2 promoter regions was observed and an increase in their protein expression levels was demonstrated by immunohistochemistry. Altogether epigenetic modifications of DNA, such as the promoter CGI demethylation of TSGs, might be related to the protective effect of soy on prostate cancer (Vardi et al., 2010). In prostate cancer cells, phenethyl isothiocyanate, a phytochemical found in large amounts in cruciferous vegetables, was reported to restore expression of silenced GSTP1 by a mechanism involving promoter demethylation and increased histone acetylation. These effects are associated with increased expression of the cyclin-dependent kinase inhibitors (CDKNs) p21 and p27, which are negative cell cycle regulators (Huang et al., 2011). Furthermore, it was established that GSTP1 gene was demethylated and reactivated following exposure to green tea polyphenols in prostate cancer cells (Pandey et al., 2010). Interestingly, lycopene also reactivated GSTP1 gene expression through reduced promoter methylation in MDA-MB-468 breast cancer cells (King-Batoon et al., 2008). In rodents, choline deficiency results in global hypomethylation of hepatic DNA and aberrant DNA hypermethylation at targeted TSG promoters such as of the GSTP1 gene promoter (Zeisel, 2012). Finally, Xiang et al. (2008) published that selenite (Se) treatment decreased general DNA methylation and caused partial promoter demethylation and re-expression of the TSGs adenomatous polyposis coli (APC) and cellular stress response 1, a gene involving tumor growth and metastasis. This study demonstrates that Se can epigenetically modulate DNA and histones to activate methylation-silenced genes. These epigenetic modifications may altogether contribute to cancer prevention by Se (Xiang et al., 2008).

CONCLUSIVE REMARKS

Considering that the discrimination power of serum prostate-specific antigen (PSA) measurement between benign and malignant tumor cells is currently under controversial discussion, the best and the most promising epigenetic marker for prostate cancer detection is the hypermethylation of GSTP1.

The previously mentioned specificity for prostate intraepithelial neoplasia allows differentiating from unmethylated benign hyperplastic prostate tissue, which always remains unmethylated. Moreover, many tumors including PCa cells shed DNA into the serum or other easily accessible body fluids (e.g., semen, urine), simplifying the detection of GSTP1 epimutations in early tumorigenesis stages. Indeed, early presence of hypermethylated TSGs does not necessarily indicate an invasive cancer, as premalignant or cancer precursor lesions can also carry these epigenetic signatures. Hence, these signatures, including miRNAs, could be used for early cancer detection in individuals with genetic predispositions or exposed to carcinogens.

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Glutathione and mitochondria

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Glutathione (GSH) is the main non-protein thiol in cells whose functions are dependent on the redox-active thiol of its cysteine moiety that serves as a cofactor for a number of antioxidant and detoxifying enzymes. While synthesized exclusively in the cytosol from its constituent amino acids, GSH is distributed in different compartments, including mitochondria where its concentration in the matrix equals that of the cytosol. This feature and its negative charge at physiological pH imply the existence of specific carriers to import GSH from the cytosol to the mitochondrial matrix, where it plays a key role in defense against respiration-induced reactive oxygen species and in the detoxification of lipid hydroperoxides and electrophiles. Moreover, as mitochondria play a central strategic role in the activation and mode of cell death, mitochondrial GSH has been shown to critically regulate the level of sensitization to secondary hits that induce mitochondrial membrane permeabilization and release of proteins confined in the intermembrane space that once in the cytosol engage the molecular machinery of cell death. In this review, we summarize recent data on the regulation of mitochondrial GSH and its role in cell death and prevalent human diseases, such as cancer, fatty liver disease, and Alzheimer's disease.

Keywords: glutathione, mitochondria, cholesterol, reactive oxygen species, steatohepatitis, Alzheimer disease

INTRODUCTION

Glutathione (GSH), the major intracellular thiol compound, is a ubiquitous tripeptide produced by most mammalian cells and it is the main mechanism of antioxidant defense against reactive oxygen species (ROS) and electrophiles. GSH (γ-glutamyl-cysteinyl-glycine) is synthesized *de novo* in two sequential enzymatic ATP-dependent reactions. In the first step, cysteine and glutamate are linked in a reaction catalyzed by the γ-glutamylcysteine synthase (γ-GCS) to form γ-glutamylcysteine. This first reaction is the rate-limiting step in the synthesis of GSH and is regulated by cysteine availability. The completion of GSH synthesis is catalyzed by glutathione synthetase (GS), in a reaction in which γ-glutamyl-cysteine is covalently linked to glycine (**Figure 1**). The antioxidant function of GSH is determined by the redox-active thiol (-SH) of cysteine that becomes oxidized when GSH reduces target molecules (Pompella et al., 2003). Upon reaction with ROS or electrophiles, GSH becomes oxidized to GSSG, which can be reduced to GSH by the GSSG reductase (GR). Thus, the GSH/GSSG ratio reflects the oxidative state and can interact with redox couples to maintain appropriate redox balance in the cell.

The synthesis of GSH from its constituent amino acids occurs exclusively in cytosol, where γ-GCS and GS reside. However, GSH is found in intracellular organelles including endoplasmic reticulum (ER), nucleus, and mitochondria to control compartment-specific needs and functions (Mari et al., 2009, 2010). Except for the ER, intracellular GSH is mainly found in its reduced form. While the percentage of the total cell GSH content found in mitochondria is minor (10–15%), the mitochondrial glutathione (mGSH) concentration is similar to that found in the cytosol. As GSH has a net negative charge at physiological pH, the high concentration of mGSH implies the existence of specific transport systems that work against an electrochemical gradient (Griffith and Meister, 1985; Garcia-Ruiz et al., 1994; Mari et al., 2009, 2010). As discussed below, despite being a small fraction of total intracellular GSH, mGSH plays a critical function in the maintenance of mitochondrial function and cell survival (Lash, 2006; Mari et al., 2013).

Mitochondria in mammalian cells generate most of the cellular energy by means of the oxidative phosphorylation (OXPHOS) that is essential for myriad cellular functions. OXPHOS provides an efficient mechanism to couple electron transport to synthesize ATP from ADP. Mitochondria are also involved in key cellular functions such as Ca^{2+} homeostasis, heme biosynthesis, nutrient metabolism (Cheng and Ristow, 2013), steroid hormone biosynthesis, removal of ammonia, integration of metabolic and signaling pathways for cell death and autophagy (Hammerman et al., 2004; Renault and Chipuk, 2013). Emerging evidence indicates a central role of mitochondria in initiating

Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; ASH, alcoholic steatohepatitis; ETC, electron transport chain; Gpx, glutathione peroxidase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; IMM, inner mitochondrial membrane; mGSH, mitochondrial glutathione; MPT, mitochondrial permeability transition; NASH, non-alcoholic steatohepatitis; NO, nitric oxide; NPC, Niemann–Pick type C disease; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation; Prx, peroxiredoxin; ROS, reactive oxygen species; SOD, superoxide dismutase; StARD1, steroidogenic acute regulatory domain 1; Trx, thioredoxin.

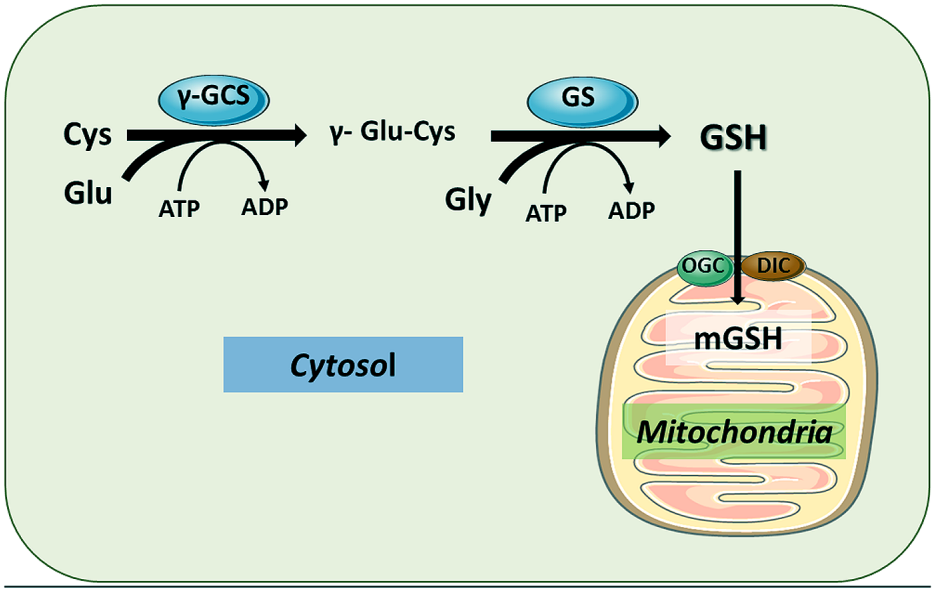

FIGURE 1 | Glutathione synthesis in cytosol and compartimentalization in mitochondria. GSH is synthesized from its constituent amino acids in the cytosol by the sequential action of γ-glutamylcysteine synthase (γ-GCS) and GSH synthase (GS). The functions of GSH are determined largely by the –SH of cysteine as by its role as a cofactor for antioxidant enzymes. Once synthesized in the cytosol, GSH can be transported to mitochondrial matrix by different carriers, particularly the 2-oxoglutarate carrier (OGC) and the dicarboxylate carrier (DIC), located in the mitochondrial inner membrane. The function the OGC has been shown to be dependent on changes in mitochondrial membrane dynamics.

signals in response to metabolic and genetic stress which affects nuclear gene expression, causing changes in cell function (Raimundo, 2014). Mitochondria contain multiple copies of their own genome, mitochondrial DNA (mtDNA), which encodes for 13 polypeptides of the OXPHOS and respiratory chain, as well as two ribosomal RNAs and 22 transfer RNAs necessary for translation of polypeptides inside mitochondria. As a consequence, the main mitochondrial proteome (∼1500 proteins) is encoded by the nucleus, translated in the cytosol and imported into the mitochondria through specific translocator complexes (TIM and TOM) of the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), respectively.

Oxidative phosphorylation is organized in a series of subsequent steps involving several redox centers distributed in five protein complexes embedded in the IMM (Sun et al., 2013; Venditti et al., 2013). Complex I obtain the electrons from NADH (NADH-coenzyme Q oxidoreductase) and complex II (succinate-coQ oxidoreductase) from succinate. Both these two complexes, independently of each other, use the lipid soluble carrier located into the IMM, ubiquinone (coenzyme Q) to form ubiquinol. From ubiquinol, the electrons pass down the redox gradient through complex III (coenzyme Q-cytochrome *c* oxidoreductase) to cytochrome *c*, then to complex IV (cytochrome *c* oxidase) and to the final acceptor, oxygen (O_2) to produce water. The fall in electron potential energy through this electron transport chain (ETC) is used to pump protons out the mitochondrial matrix to the intermembrane space (IMS). This proton pumping creates a proton-motive force consisting of electrical and proton gradients. This force is used by the fifth protein complex (Complex V, ATP synthase) to regenerate ATP from ADP. The proton-motive force created by the ETC is also used for many additional mitochondrial processes, especially those related with transport across the IMM (Kulawiak et al., 2013).

Although the primary function of mitochondria is to generate ATP as an energy molecule required for countless cell functions, a small fraction of electrons from the ETC are transferred directly to O_2, resulting in the generation of the superoxide anion, which can give rise to other ROS as well as reactive nitrogen species (RNS). Mitochondria are the primary intracellular site of oxygen consumption and the major source of ROS, most of them originating from the ETC. In accordance with this, it has been estimated that the steady-state concentration of superoxide in the mitochondrial matrix is 5- to 10- fold higher than in the cytosol (Cadenas and Davies, 2000). Associated with this constant flow of ROS generation, mitochondria are also a target for the damaging effects of oxygen radicals (Fernandez-Checa and Kaplowitz, 2005; Kaelin, 2005; Orrenius et al., 2007).

Although ROS generated under physiological conditions are not harmful, and likely play a signaling role, toxic or pathological conditions that lead to an impairment of mitochondrial function can increase the release of ROS. Mitochondrial ROS are increased under hypoxia, ischemia/reperfusion injury, chemical stress, drug treatment, and under many pathophysiological conditions (Srinivasan and Avadhani, 2012). Despite that mitochondria are exposed to the generation of oxidant species, the existence of an efficient antioxidant defense system, of which mGSH is a critical component, prevents or repairs oxidative damage generated during normal aerobic metabolism (Mari et al., 2013). In the following sections, we summarize some of the most important aspects of mGSH physiology, its role in mitochondrial function and release

of mitochondrial apoptotic factors and the impact of its depletion in disease.

MITOCHONDRIAL ROS GENERATION AND DEFENSE

Reactive oxygen species can be generated in several intracellular sites, including cytosol, peroxisomes, plasma membrane, and ER. However, mitochondrial ETC is the main cellular process of ROS generation in most cell types in physiological circumstances (Venditti et al., 2013). Although normal electron transport in mitochondria involves four-electron reduction of O_2 to water, partial reduction reactions occur under physiological conditions, causing release of superoxide anion and hydrogen peroxide (H_2O_2). Although ROS can be generated at several sites of the ETC (**Figure 2**; Brand, 2010; Quinlan et al., 2013), complex I and complex III (Venditti et al., 2013) have been shown to be the most important sources of mitochondrial superoxide generation, although significant production of ROS in complex II has recently also been reported (Quinlan et al., 2012).

The primary ROS produced by the ETC is superoxide, a free radical with moderate reactivity, whose generation can lead to more reactive or secondary ROS derivatives. Indeed, superoxide can undergo dismutation to H_2O_2, a mild oxidant that can be converted to the highly reactive hydroxyl radical in the presence of transition metals (Fe^{2+}, Cu^{+}) by means of the Fenton reaction. H_2O_2 has a longer half-life and can cross membranes (Cadenas and Davies, 2000), consequently it has been identified as a suitable second messenger molecule, in part because of its reactions with specific oxidation-prone protein cysteinyl residues (Sies, 2014), which confers properties to H_2O_2 as a mitochondrial signal (Raimundo, 2014).

Reactive oxygen species can attack biomembranes, enzymes, proteins, and nucleic acids (Venditti et al., 2013). These oxidative effects can be neutralized by antioxidant systems, engaging in a delicate balance that determines the fate and impact of ROS in cells. Although oxidative stress was defined originally as a balance between oxidants and antioxidants systems, an equilibrium among antioxidant strategies is needed to avoid the generation of oxidants and ROS (Mari et al., 2010). For instance, if the activity of superoxide scavenging by SOD2 exceeds the capacity to remove the H2O2 generated, this oxidant can cause oxidative damage or be converted to other ROS.

SUPEROXIDE, HYDROGEN PEROXIDE, AND PEROXYNITRITE GENERATION

Despite the fact that superoxide can be generated in extramitochondrial reactions, in most cell types mitochondria appear as the main source of superoxide generation. From the several sites that can generate superoxide in the mitochondrial matrix, only the superoxide produced at complex III appears to be released

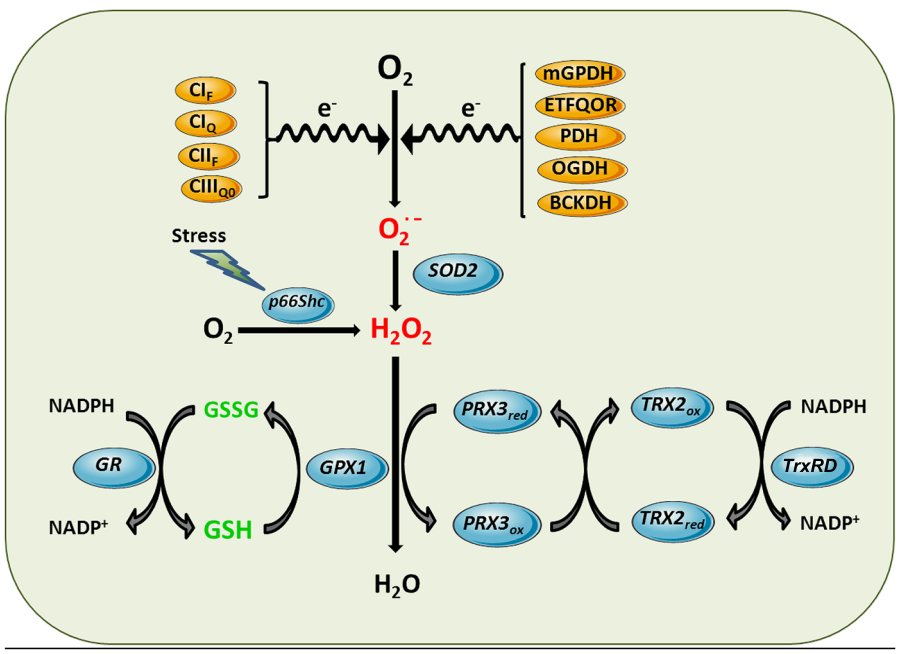

FIGURE 2 | Mitochondrial ROS generation and antioxidant defense systems. Complex I flavin site (CI_F), Complex I ubiquinone site (CI_Q), Complex II flavin site (CII_F), and Complex III_{Qo} ($CIII_{Q0}$) are sites of the ETC components shown to generate superoxide anion. Other sources of superoxide can be enzymatic reactions that transfer electrons to the ETC such as mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), and the last step of β-oxidation, electron-trasferring flavoprotein ubiquinone oxidoreductase (ETFQOR) or dehydrogenases such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) and branched-chain 2-oxoacid dehydrogenase (BCKDH). Superoxide generated in the mitochondrial matrix by these sites is dismutated to hydrogen peroxide by SOD2. Moreover, in response to stress p66Shc translocates to mitochondria to directly stimulate hydrogen peroxide generation by transferring electrons to cytochrome *c*. Hydrogen peroxide is further inactivated using the reducing equivalents of NADPH by mGSH/Gpx or Prx3/Trx2 antioxidant systems, yielding water. Mn-dependent superoxide dismutase 2 (SOD2), GSH peroxidase (GPX1), GSSG-reductase (GR), peroxiredoxin 3 (PRX3), thioredoxin-2 (TRX2), thioredoxin reductase (TrxRD).

both into the matrix and the IMS (Quinlan et al., 2013). This spatial difference (matrix vs. IMS) may determine whether mitochondrial superoxide reaches the cytosol or not. The anionic nature of superoxide and the fact that it is mostly produced in the mitochondrial matrix determine that the bulk of antioxidant defenses to neutralize superoxide and other ROS reside in the matrix. The first line of defense against superoxide is the presence of a specific member of the family of metalloenzymes called superoxide dismutases (SODs), MnSOD or SOD2, specifically located in the mitochondrial matrix, which catalyzes the dismutation of superoxide anion into H_2O_2 as shown in **Figure 2**. The dismutation of superoxide can also occur spontaneously, but such reaction is 10^4 times slower than the enzymatic dismutation by SOD2. The relevance of this enzyme is illustrated by the fact that global SOD2 deficiency leads to neonatal death in mice (Huang et al., 1997). Superoxide released into the IMS can be eliminated by a different SOD isoenzyme (Cu, Zn-SOD, or SOD1), which is found in the cytoplasm of eukaryotic cells, or scavenged by cytochrome *c* plus cytochrome *c* oxidase system (Okado-Matsumoto and Fridovich, 2001). It has been proposed that α-tocopherol can also scavenge superoxide, as suggested by experiments with submitochondrial particles isolated from mice fed with vitamin-E supplemented diet (Chow et al., 1999).

Although the dismutation of superoxide by SOD2 is a predominant source of H_2O_2, there are other reactions that directly generate H_2O_2 in mitochondria. For example, the redox activitiy of p66Shc within mitochondria has been shown to generate H_2O_2 in the absence of superoxide through oxidation of cytochrome *c* (Giorgio et al., 2005). P66Shc normally resides in the cytosol where it is involved in signaling from tyrosine kinases to Ras. However, in response to stress p66Shc translocates to mitochondria to contribute to the generation of H_2O_2. Due to the lack of unpaired electrons, H_2O_2 is not a free radical but a potent oxidant that can oxidize mitochondrial components (proteins, lipids, DNA). Besides being a potential source of more reactive free radicals via Fenton reaction, physiological generation of H_2O_2 fulfills a second messenger role and can be transported across membranes by aquaporins, a family of proteins that act as peroxiporins (Sies, 2014). The detoxification against H_2O_2 in mitochondria occurs mainly through the GSH redox system, including the glutathione peroxidases (Gpxs) and GSH reductases, as well as the presence of peroxiredoxins (Prxs; **Figure 2**) using the reducing equivalents of NADPH. Besides these antioxidant defenses that ensure H_2O_2 elimination, aquaporins have been shown to modulate mitochondrial ROS generation. In this paradigm, aquaporin 8 silencing, which is specifically expressed in IMM, enhances mitochondrial ROS generation and results in mitochondrial depolarization and cell death (Marchissio et al., 2012). In addition to these conventional sites of mitochondrial ROS generation, it has been recently reported that the branched-chain 2-oxoacid dehydrogenase (BCKDH) complex in mitochondria can produce superoxide and H_2O_2 at higher rates than complex I from mitochondria (Quinlan et al., 2014).

Peroxynitrite is a potent oxidant that is generated upon the reaction of superoxide with nitric oxide (NO). Its impact on inactivation of mitochondrial proteins depends on the level of generation in mitochondria. While ETC is the source of superoxide, the existence of mitochondrial NO synthase (mtNOS) that provides the NO required to form peroxynitrite is controversial. Although the existence of mtNOS has been described in mitochondrial fractions from different organs, recent evidence in rat liver mitochondria has questioned the existence of mtNOS, minimizing the contribution of *in situ* NO generation within mitochondrial to the formation of peroxynitrite (Venkatakrishnan et al., 2009). However, since NO is freely diffusible across membranes, it is possible that the mitochondrial production of peroxynitrite may derive from extramitochondrial NO diffusing into mitochondria to react with superoxide generated by ETC.

GLUTATHIONE REDOX CYCLE

Hydrogen peroxide is rapidly reduced to water mostly by Gpx, which utilizes the reducing equivalents from its substrate GSH. In this enzymatic reaction, GSH becomes oxidized to GSSG, which is recycled back to GSH by the NADPH-dependent GSSG reductase as shown in **Figure 2**. Since GSSG is not readily exported out of mitochondria (Olafsdottir and Reed, 1988; Yin et al., 2012), the activity of GR is an important mechanism to control the level of GSSG in mitochondria. The uncontrolled generation of GSSG during oxidative stress can contribute to mitochondrial dysfunction by glutathionylation of target proteins, as described below. The supply of NADPH is essential to regenerate GSH and dictates the rate of H_2O_2 reduction by Gpx while keeping the reduced status of mitochondria.

So far, eight isoforms of Gpx have been identified in humans, which vary in cellular location and substrate specificity (Brigelius-Flohe and Maiorino, 2013). Gpx1 is the major isoform localized in various cellular compartments, including the mitochondrial matrix and IMS (Legault et al., 2000; Mari et al., 2009), which in the liver account for about one third of the total Gpx activity (Chance et al., 1979). This selenium-containing homotetramer protein has substrate specificity for H_2O_2 and has been classically believed to be the major H_2O_2 reducing enzyme. It also has been described that γ-glutamylcysteine, the intermediate of GSH biosynthesis, is able to act as a Gpx1 cofactor in mitochondrial H_2O_2 detoxification, mimicking the physiological properties of GSH (Quintana-Cabrera et al., 2012). Surprisingly, mice with specific genetic deletion of Gpx1 appear phenotypically normal and with normal life span (Ho et al., 1997), suggesting that there are alternative compensatory mechanisms for H_2O_2 scavenging in Gpx1 deficiency. However, another report demonstrated mitochondrial stress and bioenergetics defects in GPx1 null mice (Esposito et al., 2000). Besides Gpx1, Gpx4 displays preference for lipid hydroperoxides (**Figure 3**), and hence plays a key role in protecting phospholipids, cholesteryl esters and cardiolipin and defense against apoptosis and maintenance of ETC and OXPHOS (Cole-Ezea et al., 2012). In line with this vital role in mitochondrial defense, Gpx4 null mice die during early embryonic development, while $Gpx4^{+/-}$ cells are sensitive to oxidative stress triggers (Legault et al., 2000; Muller et al., 2007). Moreover, Gpx4 has been shown recently to modulate ferroptotic

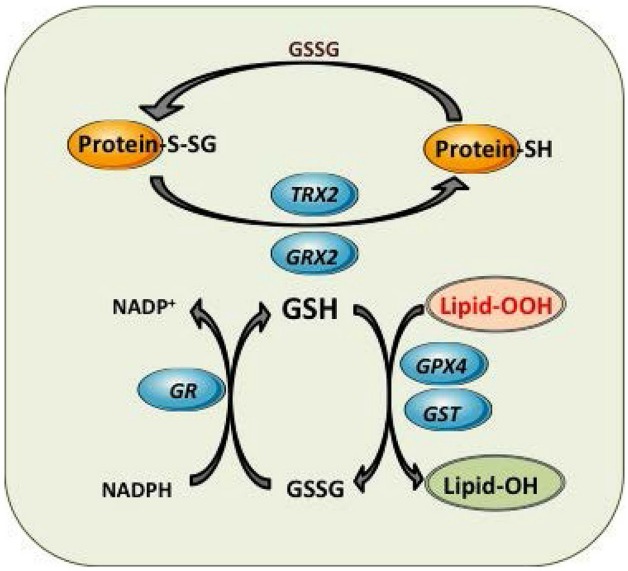

FIGURE 3 | Mitochondrial GSH redox cycle and interaction with other antioxidant defenses. Detoxification of harmful lipid peroxides (Lipid-OOH) to their corresponding hydroxides (lipid-OH) by glutathione peroxidase 4 (GPX4) and glutathione-*S*-transferases (GST). Control of mitochondrial protein glutathionylation (Prot-S-SG, Prot -SH) by glutaredoxin (GRX2) and thioredoxin 2 (TRX2).

cancer cell death, a specific form of cell death characterized by the production of iron-dependent ROS generation (Yang et al., 2014). This process involved metabolic dysfunction that results in increased production of cytosolic and lipid ROS, independently of mitochondria.

PEROXIREDOXIN–THIOREDOXIN REDOX CYCLE

Peroxiredoxins constitute a family of thiol-specific peroxidases that rely on thioredoxins (Trxs) as the hydrogen donor for the reduction of H_2O_2 and lipid hydroperoxides (Chae et al., 1999). Prx3 is the Prx isoform exclusively located in mitochondria, suggesting that it plays a primary line of defense against H_2O_2 produced by the mitochondrial respiratory chain. Prx3 homodimer has a redox-sensitive cysteine that upon reaction with H_2O_2 is oxidized to Cys-SOH, which then reacts with neighboring Cys-SH of the other subunit to form an intermolecular disulfide that can be readily reduced by thioredoxin reductase 2 (TrxRD2; Chang et al., 2004; Orrenius et al., 2007). The fact that the oxidation state of the active site cysteine of Prx can be transferred to other proteins allows Prx to function as a sensor of H_2O_2 (Rhee et al., 2012). Likewise, Prx5, the last identified member of the six mammalian Prxs (Knoops et al., 2011), is widely expressed in tissues but it is not exclusively located in mitochondria. In human cells, it has been shown that Prx5 can be targeted to mitochondria, peroxisomes, cytosol, and nucleus. The targeting of Prx5 to mitochondria is highly conserved among species (Van der Eecken et al., 2011), and it has been associated with the protection of mtDNA from oxidative attacks (Banmeyer et al., 2005). Prx5 is a peroxidase that can use cytosolic or mitochondrial Trx to reduce alkyl hydroperoxides or peroxynitrite with high rate constants, whereas its reaction with H_2O_2 is modest (Knoops et al., 2011). Therefore, as opposed to Prx3, Prx5 has been viewed mainly as a cytoprotective antioxidant enzyme rather than as a redox sensor and appears to be a unique Prx exhibiting specific functional and structural feature (Knoops et al., 2011; Zhu et al., 2012).

As noted above, Trxs are responsible for reducing Prx back to their reduced, oxidant-scavenging state, while thioredoxin reductases (TrxRDs) keep the reduced state of Trxs using NAPDH reducing equivalents, as depicted in **Figure 2**. Either Trx and TrxRD are expressed as isoforms for both predominantly cytosolic (Trx1 and TrxRD1) or mitochondrial (Trx2 and TrxRD2) localization (Enoksson et al., 2005). There are direct links between the Trx system and protein glutathionylation (Casagrande et al., 2002); and the direct reduction of mitochondrial glutaredoxin 2 (Grx2) by TrxR is also of important physiological relevance (Johansson et al., 2004; Enoksson et al., 2005). However, the reduction of the intermolecular disulfide of Prx is specific to Trx and cannot be achieved by GSH or Grx (Chang et al., 2004; Orrenius et al., 2007).

Due to their high rate constant and high abundance, Prx are thought to be responsible for scavenging nanomolar concentrations of H_2O_2 associated with redox signaling, while Gpx are likely important at higher intracellular concentrations, buffering high ROS levels to avoid cell damage and stress signaling response (Sena and Chandel, 2012). In addition there is emerging evidence indicating that both antioxidant systems (mGSH and Prx3) are mutually regulated. For instance, depletion of mGSH results in Trx2 oxidation (Zhang et al., 2007), while hypercholesterolemic pigs with selective depletion of mGSH in heart mitochondria, exhibit decreased levels of the mitochondria-specific antioxidant enzymes such as SOD2, Trx2, and Prx3 (McCommis et al., 2011). Collectively, these data highlight a key role of mGSH in maintaining a healthy antioxidant system in both systems and on H_2O_2 homeostasis.

DEFENSE AGAINST ELECTROPHILES AND PROTEIN GLUTATHIONYLATION

In addition to the defense against oxidants and ROS, GSH plays also an important role in the protection against electrophiles by glutathione-*S*-transferases (GSTs). Electrophiles can be generated as a consequence of metabolic processes involving both endogenous compounds and xenobiotics. GSTs exhibit a wide intracellular distribution, being localized in mitochondria (GSTA1), cytosol (alpha, mu, pi, and zeta) and membrane-bound (MGST1) isoforms (Aniya and Imaizumi, 2011; Li et al., 2011). Mitochondrial GSTs display both GSH transferase and peroxidase activities that detoxify harmful byproducts through GSH conjugation or GSH-mediated peroxide reduction (**Figure 3**; Hayes et al., 2005; Aniya and Imaizumi, 2011). Among human mitochondrial GSTs, the isoforms hGSTA4-4, hGSTA1, hGSTA2, and hGSTP1 showed peroxidase activity, with hGSTA4-4 exhibiting the highest activity (Gardner and Gallagher, 2001; Gallagher et al., 2006). Moreover, recent studies have shown that GSTA4 expression is selectively downregulated in adipose tissue of obese insulin-resistant C57BL/6J mice and in human obesity-linked insulin resistance (Curtis et al., 2010). Mitochondrial function

in adipocytes of lean or obese GSTA4-null mice was significantly compromised compared with wild-type controls and was accompanied by an increase in superoxide anion production.

Glutathionylation, a key mechanism of post-translational modification of proteins, involves the formation of a disulfide bridge between GSH and an available protein cysteine thiol. Non-enzymatic glutathionylation occur mostly during oxidative stress when GSH/GSSG is ∼1 and levels of ROS are high. This process is non-specific and can lead to the hyper-glutathionylation of proteins, altering their activity. Enzymatic glutathionylation reactions are tightly controlled and highly specific and are considered a major post-translational modification that occurs in response to fluctuations in local redox environments. The Grx family of proteins plays a key role in the regulation of glutathionylation reactions (**Figure 3**; Lillig et al., 2008). Although Grx are mainly responsible for deglutathionylation reactions, recent evidence has indicated a role for Grx in protein glutathionylation, mediated by the stabilization of a GSH thiyl radical which is then subsequently transferred to an available protein thiol (Starke et al., 2003). In most cases, Grx catalyze the deglutathionylation of proteins GSH disulfide mixtures (PSSGs). Grx exhibit site-specific distribution with Grx1 being specifically located in cytosol while Grx2 localizes in mitochondria. Both Grx catalyze the deglutathionylation of protein targets in two steps; first, the N-terminal cysteine on Grx deglutathionylates PSSG via a thiol disulfide exchange reaction yielding PSH and a Grx–SSG intermediate; second, Grx–SSG binds GSH and the glutathionyl moiety is removed regenerating Grx and producing GSSG. Grx2 has close to 34% homology to Grx1 and was recently identified as the enzyme required for deglutathionylation reactions in mitochondria (Gladyshev et al., 2001; Lundberg et al., 2001; Gallogly et al., 2009; Stroher and Millar, 2012). The catalytic cycle of Grx2 is also quite similar to Grx1 except that the Grx2–SSG intermediate can be reduced by NADPH and TrxRD. It is also important to point out that unlike Grx1, Grx2 complexes iron (Fe), which is required to modulate its activity (Johansson et al., 2004; Lillig et al., 2005). Interestingly, Grx2 has been shown catalyze both the deglutathionylation and glutathionylation of target proteins in mitochondria. The reversible nature of Grx2 is associated with its sensitivity to changes in GSH/GSSG; a high GSH/GSSG promotes protein deglutathionylation and a low GSH/GSSG activates Grx2 glutathionylase activity (Beer et al., 2004; Hurd et al., 2008). The main target for Grx2 in mitochondria is Complex I, although UCP3 and the 2-oxoglutarate dehydrogenase (OGDH) have been also shown to be deglutathionylated by Grx2. The role of Grx2 in maintaining mitochondrial function has been recently shown in heart from Grx2 null mice. Grx2 deletion decreased ATP production by complex I-linked substrates (Mailloux et al., 2014). $Grx2^{-/-}$ hearts also developed left ventricular hypertrophy and fibrosis and mice developed hypertension.

MITOCHONDRIA AND CELL DEATH

Besides their fundamental role in energy generation, mitochondria also play a strategic role in the regulation of cell death, including apoptosis (caspase-dependent and independent) and necrosis. Apoptosis describes a programmed mode of cell death that is characterized by a series of biochemical events that ultimately lead to cell fragmentation into compact membrane-enclosed structures, called "apoptotic bodies" that are taken up by neighboring cells and phagocytes, preventing inflammation, and tissue damage (Taylor et al., 2008). Apoptosis is induced via two main routes involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway). Both pathways are linked in some cell types by the cleavage of BID, a proapoptotic member of the Bcl-2 family of proteins, generating tBID in a process catalyzed by caspase-8 activated by the extrinsic pathways (Scaffidi et al., 1998). The intrinsic pathway of apoptosis is activated by stimuli that lead to the permeabilization of the OMM and the subsequent release of proteins from the mitochondrial IMS, such as cytochrome *c* (Martinou and Green, 2001; Kroemer et al., 2007). Cytochrome *c* normally resides within the cristae of the IMM and is sequestered by narrow cristae junctions. As mentioned above, within the IMM, cytochrome *c* participates in the mitochondrial ETC, using its heme group as a redox intermediate to shuttle electrons between complex III and complex IV. However, when the cell detects an apoptotic stimulus, such as DNA damage or metabolic stress, the intrinsic apoptotic pathway is triggered and mitochondrial cytochrome *c* is released into the cytosol (Kroemer et al., 2007). This process is thought to occur in two phases, first the mobilization of cytochrome *c* and then its translocation through permeabilized OMM. In addition to cytochrome *c*, other IMS proteins are also mobilized and released into the cytosol where they promote or counteract caspase activation and hence cell death (Li et al., 2001; Munoz-Pinedo et al., 2006). For instance, the release of Smac/Diablo into the cytosol ensures the efficiency of caspase 3 in proteolyzing target proteins through inhibition of inhibitor of apoptosis proteins (IAPs). Moreover, other specialized mitochondria-residing proteins, such as the apoptosis inducing factor (AIF) and endonuclease G, are translocated to the nuclei following their release from mitochondria and promote peripheral chromatin condensation and high molecular weight DNA fragmentation. While the above evidence indicates that the mitochondrial apoptotic pathway promotes cell death, recent provocative evidence has shown that the intrinsic apoptosis pathway mediates the pro-longevity response to mitochondrial ROS in *Caenorhabditis elegans* by triggering a unique pattern of gene expression that modulates stress sensitivity and promotes survival (Yee et al., 2014). Whether this newly described pathway has implication in mammals needs further verification.

MITOCHONDRIAL MEMBRANE PERMEABILIZATION AND RELEASE OF PROAPOPTOTIC PROTEINS

While mitochondrial proteins are normally secured in the IMS, understanding the mechanism of release may be of relevance to control cell death. The rupture of the physical barrier (OMM) that limits their release into the cytosol constitutes a point-of-no-return in cell death (Martinou and Green, 2001; Kroemer et al., 2007). Current evidence supports the existence of two compatible mechanisms leading to the breakage of OMM: the mitochondrial permeability transition (MPT), and the permeabilization of OMM without disruption of the inner membrane. The former

is a process characterized by mitochondrial swelling, IMM permeabilization and OMM rupture as a secondary event. On the other hand, there is evidence indicating the selective permeabilization of OMM in the absence of disrupted inner membrane. The relative prevalence of these pathways in the regulation of cell death is not definitively established. One important feature of mitochondrial permeabilization is the loss of function resulting in the inability of mitochondria to synthesize ATP through the OXPHOS. However, while the final outcome of mitochondrial dysfunction is cell death, the phenotype of death (apoptosis and/or necrosis) depends on the level of cellular ATP, as ATP is required for the efficient assembly of the apoptosome. Alternatively to MPT in the control of OMM permeabilization, Bcl-2 family members are also known to play a major function. Bcl2-family death agonists induce OMM permeabilization, thereby promoting cytochrome *c* release, whereas Bcl2-family death antagonists prevent it. Thus, Bcl2-family proteins control mitochondrial integrity, regulate cytochrome *c* release and intrinsic apoptosis (Youle and Strasser, 2008). Under non-apoptotic conditions, Bax is inactive and present in the cytosol as a monomer. Following an apoptotic stimulus, Bax is activated and translocates to the mitochondria, where it undergoes a conformational change and inserts into the OMM. Bax oligomerization is associated with the formation of openings in the OMM to allow the release of cytochrome *c* and other IMS proteins into the cytosol, and hence Bax oligomerization is considered a critical regulatory point in cell death (Youle and Strasser, 2008). Further understanding the mechanisms underlying OMM permeabilization may provide novel strategies to regulate cytochrome *c* and control apoptosis.

REGULATION OF CELL DEATH BY mGSH

As opposed to apoptosis, necrosis is a morphologically distinct form of cell death responsible for irreversible tissue destruction due to bioenergetic failure and oxidative damage. The fundamental difference relative to apoptosis is the rapid loss of cellular membrane potentials due to energy depletion and ion pump/channel failures, leading to swelling, rupture, and cytolysis. MPT is a regulated non-selective water and solute-passing protein complex whose molecular characterization remains elusive. Available evidence suggests a role for voltage-dependent anion channel (VDAC), located in the OMM, and adenine nucleotide translocase (ANT) across the IMM (Kroemer et al., 2007; Baines, 2010) and the translocator protein TSPO [previously called peripheral benzodiazepine receptor (PBR)] as components of MPT. However, liver mitochondria from mice lacking ANT1 and ANT2 can still undergo Ca^{2+}-induced swelling and MPT, although at a higher threshold, which has been interpreted as evidence against a role for ANT in MPT (Kokoszka et al., 2004). However, recent evidence has demonstrated that TSPO is dispensable for MPT (Sileikyte et al., 2014). In particular, heart mitochondria from mice with selective TSPO deletion in hearts undergo MPT and are as sensitive to ischemia–reperfusion injury as hearts from control mice. In contrast, the prolyl isomerase cyclophilin D in the mitochondrial matrix is an essential regulator of MPT and the only genetically proven indispensable MPT component (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Upon oxidative stress, sudden MPT causes massive ion influx that dissipates mitochondrial membrane potential and shuts down OXPHOS, ATP production and ROS overgeneration. Concomitantly, water influx causes matrix swelling, rupture of the rigid OMM and release of apoptogenic proteins sequestered in IMS although, apoptotic cell death under MPT is inhibited due to energetic failure and ATP exhaustion and oxidative stress-mediated caspase inactivation.

A critical step in mitochondrial apoptosis is the mobilization of cytochrome *c* from IMS. It has been proposed that during mobilization cytochrome *c* detaches from the IMM and dissociates from the membrane phospholipid cardiolipin. A significant proportion of the cytochrome *c* in the mitochondria seems to be associated with cardiolipin, involving two major mechanisms. At physiological pH, cytochrome *c* has a net positive charge (+8), establishing an electrostatic bond with the anionic cardiolipin (Gonzalvez and Gottlieb, 2007). In addition, cytochrome *c* has a hydrophobic channel through which one of the four-acyl chains of cardiolipin inserts. The other chains of cardiolipin remain in the membrane, thereby anchoring cytochrome *c* to the IMM. One mechanism that contributes to cytochrome *c* detachment from IMM involves cardiolipin oxidation because oxidized cardiolipin has a much lower affinity for cytochrome *c* than the reduced form. Cardiolipin can be oxidized by ROS or by the cardiolipin–cytochrome *c* complex (Kagan et al., 2005). Detachment of cytochrome *c* from cardiolipin might also be triggered by increased cytosolic calcium, which weakens the electrostatic interaction between cytochrome *c* and cardiolipin and further generates ROS via MPT.

In addition, it has been described that oxidized cardiolipin modulates the biophysical properties of OMM to allow oligomerized Bax to insert and permeabilize the OMM (Mari et al., 2008; Montero et al., 2010; Landeta et al., 2011). Since mitochondrial ROS contribute to cardiolipin oxidation and are controlled by antioxidants (Mari et al., 2008, 2009), mGSH arises as an important modulator of apoptotic cell death by indirectly controlling the redox state of cardiolipin (Mari et al., 2008; Montero et al., 2010). mGSH not only regulates cell death susceptibility but the outcome of cell death (necrosis or apoptosis). Thiol redox status regulate MPT and enhanced ROS generation can target critical cysteine residues in cyclophilin D, implying that mGSH depletion would favor MPT via redox pathways targeting MPT components. In addition, through modulation of cardiolipin redox state, mGSH can also regulate OMM permeabilization via MPT and the release of apoptogenic proteins.

GLUTATHIONE IMPORT TO MITOCHONDRIA

As indicated above, despite the fact that the concentration of mGSH is high, GSH is not synthesized *de novo* in the mitochondrial matrix, as this organelle lacks the enzymes required for GSH synthesis. Furthermore, GSH has an overall negative charge at physiological pH and mitochondria exhibit a large negative membrane potential. Moreover, although GSH can cross OMM, its transport into mitochondrial matrix cannot be explained by simple diffusion. Therefore, mGSH arises from the cytosol GSH by the activity of specific carriers (**Figure 1**; Griffith and Meister, 1985).

Accordingly, recent findings using dynamic oxidant recovery assays and GSH-specific fluorescent reporters, established that free communication of GSH pools exists between cytosol and IMS. In contrast, no appreciable communication was observed between the GSH of the IMS and matrix (Kojer et al., 2012). Based on substrate specificity, potential candidates to transport GSH into mitochondria have been identified, including the 2-oxoglutarate carrier (OGC; SLC25A11) and the dicarboxylate carrier (DIC; SLC25A10; Chen and Lash, 1998; Chen et al., 2000; Coll et al., 2003; Wilkins et al., 2012), mainly in kidney and liver, and tricarboxylate carrier (TTC, SLC25A1) in brain mitochondria and astrocytes (Wadey et al., 2009). OGC imports cytosolic GSH into mitochondria in exchange for 2-oxoglutarate (2-OG) and other dicarboxylates. Instead, DIC mediates electro-neutral exchange of dicarboxylates or GSH for inorganic phosphate (Mari et al., 2009). The relative contribution of each system is different depending on the cell type, as discussed in the following section.

TISSUE-SPECIFIC FEATURES OF GLUTATHIONE IMPORT TO MITOCHONDRIA

Previous studies in a renal proximal tubular cell line, NRK-52E, indicated that overexpression of OGC and DIC increased mGSH levels and protected against oxidant-mediated cell death (Lash et al., 2002; Xu et al., 2006). Similar findings were recently reported in primary renal proximal tubular cells from uninephrotectomized rats (Benipal and Lash, 2013). The findings with DIC overexpression in kidney cells indicated a role for this carrier in the mitochondrial transport of GSH in exchange with inorganic phosphate. In contrast to kidney, no clear evidence for DIC in the transport of mitochondrial GSH was found in rat liver (Coll et al., 2003). The functional expression in *Xenopus laevis* oocytes microinjected with the DIC cRNA from rat liver did not result in significant GSH transport activity (Coll et al., 2003). Moreover, in contrast to rat kidney mitochondria, the import of GSH in rat liver mitochondria showed both a high affinity and low affinity transport component (Martensson et al., 1990). Likewise, kinetic analyses of 2-oxoglutarate transport in rat liver mitochondria indicated the presence of a single Michaelis–Menten component with kinetic parameters in the range of those reported previously for kidney mitochondria (Chen et al., 2000; Coll et al., 2003). These findings suggest that the OGC accounts for the low-affinity high capacity of GSH transport in liver mitochondria, and imply that the nature of the high affinity GSH transporter remains to be identified. Also, OGC and DIC together accounted for only an apparent 45–50% of the total GSH uptake in liver mitochondria, in contrast to 70–80% described in kidney mitochondria (Zhong et al., 2008).

Interestingly, it has been suggested that Bcl-2 participates as a regulator of mGSH transport by modulating the affinity of OGC for GSH (Wilkins et al., 2012). Bcl-2 and OGC appear to act in a coordinated manner to increase the mGSH pool and to enhance the resistance of neurons to mitochondrial oxidative stress. In line with this outcome, stable motoneuron-like cell lines overexpressing OGC displayed an increased expression of Bcl-2 protein, an effect that was dependent on the mGSH increase. Conversely, a knockdown of Bcl-2 provoked a decrease in mGSH and a concomitant oxidative stress sensitization (Wilkins et al., 2014). Therefore, the antioxidant-like and antiapoptotic function attributed to Bcl-2 could, at least in part, depend on its potential to regulate the mGSH transport and status.

In brain, the properties of GSH transport in isolated rat brain mitochondria seemed to be different from those reported previously for kidney mitochondria, as they were influenced most by inhibitors of the tricarboxylate carrier, citrate, isocitrate, and benzenyl-1,2,3-tricarboxylate (Wadey et al., 2009). Moreover, in mouse brain mitochondria another study showed that OGC and DIC are both expressed in cortical neurons and astrocytes (Kamga et al., 2010). In addition, butylmalonate, an inhibitor of DIC, significantly decreased mGSH, suggesting DIC as the major GSH transporter in mouse cerebral cortical mitochondria (Kamga et al., 2010). It has been shown that pharmacological inhibition or knockdown of a single mGSH transporter significantly sensitized neurons to oxidative and nitrosative stress (Wilkins et al., 2013). Interestingly, a role for UCP2 in the transport of mGSH has been described in neurons, suggesting that the transport of protons back into the matrix by UCP2 may favor the movement of GSH (de Bilbao et al., 2004). These studies suggest that multiple IMM anion transporters might be involved in mGSH transport and that they might differ in different cell populations within the brain. These findings indicate that mGSH levels and its transport are major determinants in brain cell susceptibility to oxidative stress, although little is known about the regulation of the mGSH transport in brain.

MITOCHONDRIAL MEMBRANE PROPERTIES AND IMPACT ON GLUTATHIONE IMPORT

Previous studies in liver mitochondria have revealed that membrane dynamics regulate the transport of mGSH. Membrane physical properties are mainly regulated by fatty acid composition and the cholesterol/phospholipid molar ratio (Coll et al., 2003; Lluis et al., 2003; Ikonen, 2008). Parallel to the findings from rat liver mitochondria, it has been recently reported that mitochondrial cholesterol enrichment, resulting in mGSH depletion, is a major mechanism of anthrax lethal toxin-induced macrophage cell death (Ha et al., 2012). Mitochondria are cholesterol-poor organelles compared to plasma membrane, and this regulated transport of cholesterol in mitochondria plays physiological role in the synthesis of bile acids in liver and steroidogenic hormones in other tissues. (Garcia-Ruiz et al., 2009; Montero et al., 2010). Consistent with the role of cholesterol in the regulation of membrane dynamics, cholesterol loading in mitochondrial membrane results in increased membrane order parameter and in the reduction in the activity of specific membrane carriers, i.e., GSH transport system without effect on other transporters, indicating that the impact of changes in membrane dynamics on carrier function is not universal (Fernandez et al., 2009a; Ha et al., 2012). Moreover, functional expression studies in *X. laevis* oocytes demonstrated that the OGC is sensitive to increased membrane order caused by cholesterol loading (Coll et al., 2003). Thus, cholesterol regulates the transport of mGSH, which in turn, modulates susceptibility to oxidative stress and cell death, therefore emerging as an important target in pathophysiology of diverse diseases such as steatohepatitis (SH) or Alzheimer's disease (AD;

Figure 4; Krahenbuhl et al., 1995; Armstrong and Jones, 2002; Garcia-Ruiz et al., 2002; Fernandez-Checa and Kaplowitz, 2005; Lluis et al., 2005, 2007; Mari et al., 2006, 2008, 2009; Lu and Armstrong, 2007; Fernandez et al., 2009b; Fernandez-Checa et al., 2010).

Based on the above findings, understanding the regulation of mitochondrial cholesterol trafficking may be of potential relevance in cell death regulation and disease progression. Given its lipophilic properties and water insolubility, non-vesicular transport by specific carriers stands as the major mechanism of cholesterol transport between organelles. In particular, mitochondrial cholesterol transport is preferentially regulated by the steroidogenic acute regulatory domain 1 (StARD1), the founding member of a family of lipid transporting proteins that contain StAR-related lipid transfer (START) domains (Miller, 2013). StARD1 is an OMM protein which was first described and best characterized in steroidogenic cells where it plays an essential role in cholesterol transfer to the IMM for metabolism by cholesterol side chain cleavage enzyme (CYP11A1) to generate pregnenolone, the precursor of steroids. Pregnenolone synthesis in mitochondria is limited by the availability of cholesterol in the IMM (Clark, 2012). Despite similar properties with StARD1, other StART members cannot replace StARD1, as germline StARD1 deficiency is lethal due to adrenocortical lipoid hyperplasia (Caron et al., 1997). For instance, targeted mutations in MLN64 (StARD3), another START member with wide tissue distribution, have been shown to cause minor alterations in metabolism and intracellular distribution of cholesterol, questioning its contribution to intramitochondrial cholesterol trafficking (Kishida et al., 2004; Miller, 2007). StARD1 activation and regulation is complex and poorly understood. Its activation is regulated at the transcriptional and post-translational levels, as StARD1 phosphorylation at serine194 has been shown to enhance the trafficking of cholesterol to IMM in murine steroidogenic cells, resulting in increased steroidogenesis (Arakane et al., 1997; Kil et al., 2012). Moreover, the role of ER stress in the regulation of StART family members has been limited to StARD5 with conflicting results reported for StARD4. However, recent data have provided evidence that ER

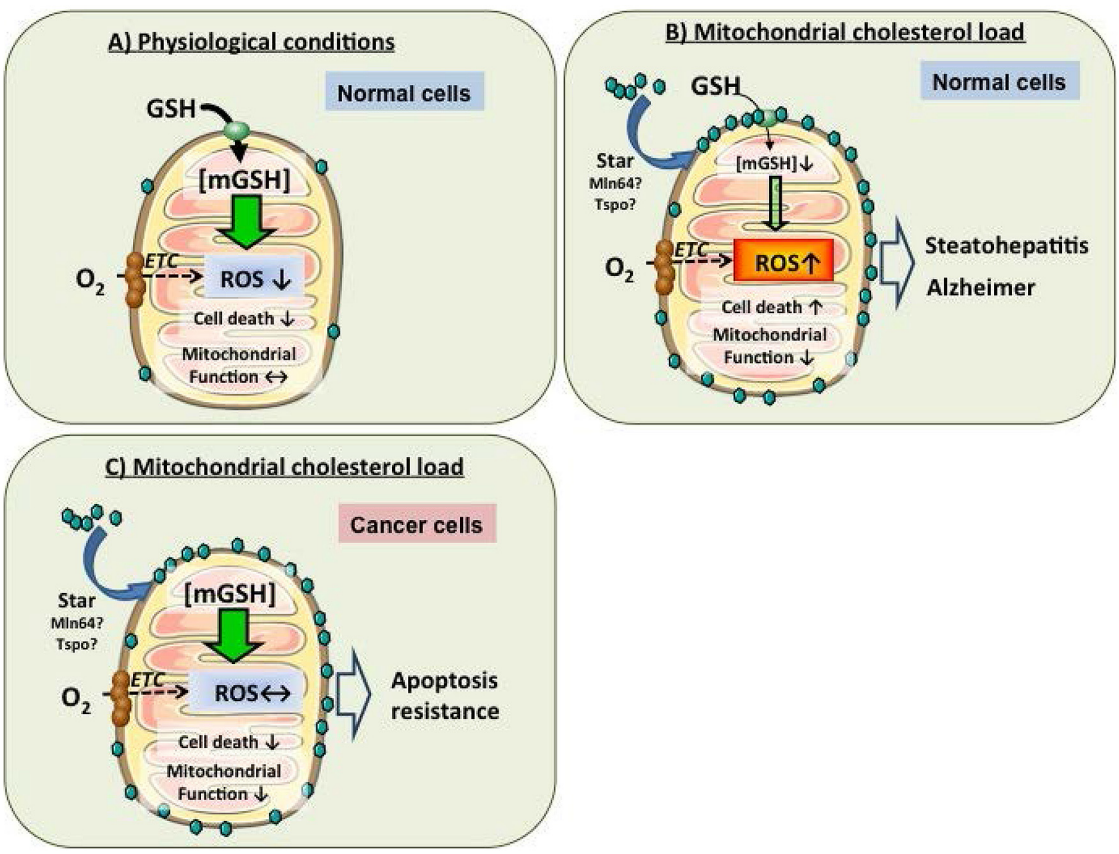

FIGURE 4 | Regulation of mGSH by mitochondrial cholesterol loading in health and disease. (A) In healthy mitochondria, mGSH levels can cope with the ROS generated in physiological conditions, avoiding cell death induction and maintenance of mitochondrial functions. **(B)** In cholesterol-enriched mitochondria, the mGSH transport is impaired resulting in mGSH depletion. mGSH levels below a certain threshold compromise ROS detoxification, leading to oxidative stress and resulting ultimately in higher susceptibility to cell death. **(C)** However, cancer cells exhibit increased cholesterol enrichment of mitochondrial membrane but paradoxically maintain mGSH levels despite changes in membrane physical properties by an as yet uncharacterized mechanism. Increased mitochondrial cholesterol and mGSH protect against mitochondrial membrane permeabilization and cell death. Thus, targeting mitochondrial cholesterol or mGSH may be a novel approach in the treatment of cancer.

stress induces the transcriptional upregulation of StARD1 independently of SREBP regulation (Fernandez et al., 2013). High cholesterol feeding caused the repression of SREBP-2 regulated genes, HMG-CoA reductase, but not that of StARD1. Similar findings have been reported in brain mitochondria in a murine model of AD (Barbero-Camps et al., 2014). Furthermore, the increase in mitochondrial cholesterol in brain mitochondria of AD was not accompanied by a selective increase in mitochondrial-associated membranes (MAMs), corresponding to the contact between ER and mitochondria, suggesting that StARD1-mediated cholesterol trafficking to mitochondria is independent of MAM, a specific membrane domain made of ER and mitochondria bilayers, which is thought to be of relevance in the traffic of lipids. TSPO, a protein particularly abundant in steroidogenic tissues and primarily localized in the OMM, has been suggested to play an important role in steroidogenesis via the transport of cholesterol to the IMM (Papadopoulos and Miller, 2012; Miller, 2013). However, quite interestingly, recent studies using tissue-specific genetic deletion of TSPO demonstrated that TSPO is dispensable for steroidogenesis in Leydig cells (Morohaku et al., 2014), questioning the relevance of previous findings on TSPO using pharmacological ligands and inhibitors. These data underscore that TSPO does not play a significant role in the trafficking of cholesterol to IMM, and highlights the relevance of StARD1 in this process.

Finally, a role for caveolin-1 (CAV1) in mitochondrial cholesterol has been recently reported. CAV1 is a key component of caveolae, specialized membrane domains particularly enriched in cholesterol and sphingolipids, and CAV is known to bind cholesterol with high affinity (Murata et al., 1995; Pol et al., 2005; Boscher and Nabi, 2012). CAV's ability to move between cell compartments, mitochondria–ER and plasma membrane, might contribute to regulation of cholesterol fluxes and distributions within cells (Pol et al., 2001, 2005; Parton and Simons, 2007; Bosch et al., 2011). In line with these features, CAV1 deficiency has been shown to increase mitochondrial cholesterol in hepatocytes causing perturbations in mitochondrial membrane dynamics and function, and as expected, mGSH depletion (Bosch et al., 2011). The mitochondrial dysfunction sensitizes CAV1 null mice to SH and neurodegeneration. Whether the trafficking of mitochondrial cholesterol in the absence of caveolin-1 occurs via MAM or StARD1 remains to be further investigated.

ROLE OF MITOCHONDRIA AND MITOCHONDRIAL GSH IN DISEASE

Given the role of mitochondria in oxygen consumption, metabolism and cell death regulation, alterations in mitochondrial function or dysregulation in cell death pathways contribute to many diseases such as cancer, SH, or neurodegeneration. Consistent with its role in regulating mGSH, mitochondrial cholesterol accumulation emerges as a key factor regulating ROS and electrophile detoxification, and hence disease progression by sensitizing to secondary hits such as TNF, hypoxia or toxic amyloid peptides. In the following sections we will briefly cover examples of diseases where mitochondria cholesterol, oxidative stress, and mGSH depletion have been shown to play a role, such as cancer, fatty liver disease, and AD.

CANCER BIOLOGY AND THERAPEUTICS

Cancer cells exhibit critical metabolic transformations induced by mutations in oncogenes (gain-of-function) and tumor suppressor genes (loss-of-function) that result in cell deregulation associated with enhanced cellular stress. Adaptation to this stress phenotype is required for cancer cells to survive and involves the participation of genes that regulate generation and sensitization to ROS-mediated cell death. In this context, small molecules that selectively kill cancer cells are a promising approach for the treatment of cancer. Experiments using a cell-based small-molecule screening and quantitative proteomics, revealed the potential of piperlongumine, a natural product isolated from the plant species *Piper longum L*, as a cytotoxic agent triggering apoptosis and necrosis in leukemia cells (Bezerra et al., 2007). Moreover, piperlongumine induces ROS generation resulting in the killing of transformed cells *in vitro* and *in vivo* but not primary normal cells (Raj et al., 2011). Piperlongumine leads to decreased GSH and increased GSSG levels in cancer cells without effects in non-transformed cells, and these effects paralleled the ability of piperlongumine to cause alterations in mitochondrial morphology and function. Consequently, co-treatment with piperlongumine and *N*-acetyl-L-cysteine (NAC) prevented piperlongumine-mediated GSH depletion and cell death in cancer cells. These findings support the concept that normal cells have low basal levels of ROS and a diminished reliance on the ROS stress-response, while cancer cells have high levels of ROS, and hence, are expected to have a strong reliance on the ROS stress-response pathway. In line with the relationship between ROS and cancer it has been suggested that antioxidants may protect against cancer. However, randomized clinical trials have produced inconsistent results and some studies indicated that antioxidants increase cancer risk (van Zandwijk et al., 2000; Klein et al., 2011; Watson, 2013). A recent study in oncogene-induced lung cancer demonstrated that treatment with NAC and vitamin E accelerate cancer progression, stimulating cell proliferation by reducing ROS, DNA damage, and p53 expression (Sayin et al., 2014). The use of small molecules that alter the levels of ROS such as β-phenethyl isothiocyanate (PEITC), buthionine sulphoximine, curcumin, or 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid (CDDO) derivatives, has been suggested for the treatment of cancer by promoting ROS generation and GSH depletion in cancer cells (Schumacker, 2006; Trachootham et al., 2006; Yue et al., 2006; Ravindran et al., 2009). Interestingly, mGSH depletion has also been associated with apoptosis or autophagy induced by chemotherapeutic drugs. For instance, the novel triterpenoid methyl CDDO derivative (CDDO-Me) potently induced cytotoxicity in imatinib-resistant myeloid leukemia cells, accompanied by a rapid and selective depletion of mGSH resulting in increased generation of ROS and mitochondrial dysfunction (Samudio et al., 2005, 2008). Moreover, PEITC caused a rapid depletion of mGSH and a significant elevation of ROS and NO, induced a disruption of the mitochondrial electron transport complex I, and a significant suppression of mitochondrial respiration that resulted in cytotoxicity in leukemia cells (Chen et al., 2011).

As mGSH is regulated by cholesterol, as described above, the trafficking of mitochondrial cholesterol may modulate cancer cell biology. Cholesterol metabolism is deregulated in tumors,

which exhibit a paradoxical stimulation in *de novo* cholesterol synthesis despite hypoxia-mediated downregulation of HMG-CoA reductase by hypoxia (Nguyen et al., 2007; Garcia-Ruiz et al., 2009). In addition to its continued synthesis, cholesterol trafficking to mitochondria has been reported in tumor cells, including in mitochondria from hepatocellular carcinoma (HCC) due to overexpression of StARD1 (Montero et al., 2008). Mitochondrial cholesterol loading in cancer cells may actually account for the recognized mitochondrial dysfunction and resistance to Bax-mediated cell death induced by chemotherapy agents. In line with this hypothesis, treatments that resulted in mitochondrial cholesterol loading in tumor cells impaired stress-induced apoptosis (Lucken-Ardjomande et al., 2008; Montero et al., 2008), while StARD1 knockdown or treatments that resulted in downregulation of cholesterol loading sensitized HCC cells to chemotherapy. These findings identify the mitochondrial cholesterol loading in cancer cells, particularly HCC, as a mechanism contributing to chemotherapy resistance and evasion of Bax-mediated apoptosis (**Figure 4**). While mitochondrial cholesterol depletes mGSH due to impaired transport via OGC in primary hepatocytes and in SH, cancer cells paradoxically maintain mGSH homeostasis by a still ill-defined mechanism that is under investigation.

ALCOHOLIC AND NON-ALCOHOLIC FATTY LIVER DISEASE

Fatty liver disease represents a spectrum of liver disorders that begins with simple steatosis. This intial stage can progress to SH and culminate in cirrhosis and liver cancer. SH is an intermediate stage of fatty liver disease and one of the most common causes of chronic liver disease worldwide that may progress to cirrhosis and liver cancer. SH is characterized by steatosis, oxidative stress, hepatocellular death, inflammation and fibrosis and encompasses alcoholic (ASH) and non-alcoholic steatohepatitis (NASH). Unfortunately, there is no approved therapy for ASH/NASH, which reflects our incomplete understanding of the underlying mechanisms (Tilg and Diehl, 2000; Angulo and Lindor, 2002; Brunt, 2004). The development of steatosis in ASH/NASH is secondary to the metabolic disturbances in ASH and NASH, including insulin resistance, adipose tissue lipolysis, stimulation of *de novo* lipid synthesis and impaired mitochondrial fatty acid oxidation (Garcia-Ruiz and Fernandez-Checa, 2006; Garcia-Ruiz et al., 2011, 2013a,b). A key concept in SH pathogenesis is the two-hit hypothesis, which posits that hepatic steatosis sensitizes fatty liver to secondary hits, such as inflammatory cytokines and oxidative stress. However, recent evidence has shown that the type rather than the amount of fat plays a critical role in the transition from steatosis to ASH/NASH. In line with this hypothesis, previous studies have shown that chronic alcohol feeding in various models results in the depletion of mGSH due to cholesterol loading in mitochondria (Garcia-Ruiz et al., 1994; Colell et al., 1997, 1998, 2001; Zhao et al., 2002; Garcia-Ruiz and Fernandez-Checa, 2006) and that strategies aimed to correct the loss of mitochondrial membrane fluidity restore the mitochondrial transport of GSH and replenish the mGSH pool in alcohol-fed models. Moreover, recent evidence in rats fed an ethanol-polyunsaturated fatty acid treatment confirmed the mitochondrial cholesterol accumulation and GSH depletion, leading to SH, and these effects were prevented by betaine treatment (Varatharajalu et al., 2014). Altered alcohol-induced ER stress involves alterations in the methionine cycle and hyperhomocysteinemia, and treatment with betaine prevents alcohol-induced ER stress, steatosis, and liver injury (Ji and Kaplowitz, 2003, 2004). Moreover, tauroursodeoxycholic acid, a chemical chaperone shown to prevent ER stress (Ozcan et al., 2006), restored the mGSH pool in alcohol fed rats (Colell et al., 2001) and blocked alcohol-induced ER stress (Fernandez et al., 2013). The mechanisms of alcohol-induced mitochondrial cholesterol trafficking, mediated by alcohol-induced upregulation of StARD1, requires alcohol-induced acid sphingomyelinase activation (Fernandez et al., 2013).

Increased cholesterol synthesis and levels have been reported in liver biopsies from patients with NASH (Puri et al., 2007; Caballero et al., 2009) and mGSH depletion has been observed in models and patients with NASH (Serviddio et al., 2008). This outcome is consistent with the increased expression of StARD1 in patients with NASH but not with simple steatosis (Caballero et al., 2009). In line with these findings, recent data reported that the inhibition of microsomal triglyceride transfer protein, a model of liver steatosis, induced the increase in free cholesterol in mitochondria resulting in mGSH depletion (Josekutty et al., 2013). In addition to mGSH depletion, NASH is also characterized by impaired SOD2 activity, which may contribute the increased generation of mitochondrial superoxide and subsequent peroxynitrite levels that target mitochondrial proteins causing their inactivation. In principle, strategies such as SOD mimetics aimed to improve SOD2 activity may be of relevance in NASH. However, the use of SOD mimetics in parallel with the reported mGSH depletion can cause increased H_2O_2 and overall oxidant-dependent liver injury (Montfort et al., 2012). This scenario implies that the combination of SOD mimetics and mGSH replenishment may more efficient in NASH treatment.

ALZHEIMER DISEASE

Alzheimer disease is a major neurodegenerative disorder and the main cause of adult dementia. The main risk factor for AD is aging and therefore the number of people worldwide facing AD development increases every year. AD is characterized by progressive memory loss, cognitive impairment and disruption of synaptic plasticity. Although there are recommended therapies for AD, such as acetylcholinesterase inhibitors and the *N*-methyl-D-aspartate receptor antagonists, they are inefficient and do not prevent disease progression, reflecting our incomplete understanding of AD pathogenesis. Experimental models and human data established two main theories underlying AD, the accumulation of toxic amyloid β (Aβ) peptides, characteristic of senile plaques, and the aggregation of tau protein, a microtubule-associated protein expressed in neurons that is involved in the stabilization of microtubules in the cytoskeleton. The pathogenic processing of the amyloid precursor protein (APP) leads to toxic Aβ generation and is considered a critical mechanism of AD. Accordingly, a coding mutation (A673T) in APP has been recently shown to protect against AD and age-related cognitive decline in elderly Icelanders (Jonsson et al., 2012). This substitution, which is close to the aspartyl protease

β-site in APP, reduces the formation of amyloidogenic peptides *in vitro* by 40%. The protective effect of the A673T substitution against AD provides strong evidence for the hypothesis that reducing the β-cleavage of APP may protect against the disease. Amiloidogenic processing of APP yields toxic Aβ peptides. In this pathway, the β- and γ-secretases cleave APP at the N- and C-termini of the Aβ peptide, respectively. β-Secretase has been characterized as a membrane-bound aspartic protease termed beta-site APP-cleaving enzyme 1 (BACE1), while γ-secretase is a complex comprised of presenilin-1 or -2, nicastrin, anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2; Haass, 2004). Another novel member of the γ-secretase complex has been identified. β-arrestin 2 physically associates with the Aph-1α subunit of the γ-secretase complex and redistributes the complex toward detergent-resistant membranes, increasing the catalytic activity of the complex (Thathiah et al., 2013). Moreover, β-arrestin 2 expression is elevated in individuals with AD and its overexpression leads to an increase in Aβ peptide generation, whereas genetic silencing of Arrb2 (encoding β-arrestin 2) reduces generation of Aβ in cell cultures and in $Arrb2^{-/-}$ mice. In addition to its amyloidogenic processing by β- and γ-secretases, APP can be cleaved within the Aβ domain by α-secretase. This non-amyloidogenic processing prevents the deposition of intact Aβ peptide and results in the release of a large soluble ectodomain, sAPPα, from the cell, which has neuroprotective and memory-enhancing effects. Members of the ADAMs, a disintegrin and metalloprotease family of proteases, have been shown to possess α-secretase activity (Hooper and Turner, 2002). The pathogenic processing of APP into toxic Aβ fragments occurs in cholesterol-enriched membrane domains of the plasma membrane, known as lipid rafts, consistent with the recognized role of cholesterol in AD pathogenesis based upon experimental and epidemiological evidence linking plasma cholesterol levels and AD development (Notkola et al., 1998; Wolozin et al., 2000; Anstey et al., 2008). High cholesterol levels correlated with Aβ deposition and the risk of developing AD, while patients taking the cholesterol-lowering drug statins were found to have a lower incidence of the disease (Notkola et al., 1998; Wolozin et al., 2000). Exploiting the relative detergent insolubility of lipid rafts, there has been evidence indicating the localization of APP, the α-, β- and γ-secretases in rafts (Wahrle et al., 2002; Vetrivel et al., 2005). In addition, the activities of BACE1 and γ-secretase are stimulated by lipid components of rafts, such as glycosphingolipids and cholesterol (Sawamura et al., 2004; Kalvodova et al., 2005; Ariga et al., 2008; Osenkowski et al., 2008). Besides its extracellular deposition, current evidence indicates the processing and targeting of APP and Aβ to intracellular sites, including mitochondria (Lin and Beal, 2006). Moreover, levels of mitochondrial APP are higher in affected brain areas and in subjects with advanced disease symptons (Devi et al., 2006). Immunoelectron microscopy analyses indicated the association of APP with mitochondrial protein translocation components, TOM40 and TIM23, which correlated with decreased import of respiratory chain subunits *in vitro*, decreased cytochrome oxidase activity, increased ROS generation and impaired mitochondrial reducing capacity (Devi et al., 2006). Although the molecular mechanisms of mitochondrial Aβ targeting remains poorly understood, Aβ stimulates mitochondrial ROS generation, contributing to Aβ toxicity in neurons (Behl et al., 1994; Casley et al., 2002; Lustbader et al., 2004). In addition to the amyloidogenic effect of cholesterol by fostering Aβ generation from APP, recent data has provided evidence that mitochondrial cholesterol accumulation sensitizes neurons to Aβ-induced neuroinflammation and neurotoxicity by depleting mGSH, effects that are prevented by mGSH replenishment (Fernandez et al., 2009b). The mechanism of mitochondrial cholesterol accumulation involves the upregulation of StARD1 induced by Aβ *via* ER stress (Barbero-Camps et al., 2014), confirming previous findings in hepatocytes (Fernandez et al., 2013). Although not reported in patients, the trafficking of cholesterol to mitochondria may be of clinical relevance to human AD due to the described enhanced expression of StARD1 in pyramidal hippocampal neurons of AD-affected patients (Webber et al., 2006). Moreover, a novel mouse model engineered to have enhanced cholesterol synthesis by SREBP-2 overexpression superimposed to APP/PS1 mutations triggered Aβ accumulation and tau pathology (Barbero-Camps et al., 2013). This triple transgenic model exhibited increased mitochondrial cholesterol loading and mGSH depletion and accelerated Aβ generation by β-secretase activation compared to APP/PS1 mice (Barbero-Camps et al., 2013). Moreover, SREBP-2/APP/PS1 mice displayed synaptotoxicity, cognitive decline, tau hyperphosphorylation and neurofibrillary tangle formation in the absence of mutated tau, indicating that cholesterol, particularly mitochondrial cholesterol, can precipitate Aβ accumulation and tau pathology. Importantly, *in vivo* replenishment of mGSH with cell- permeable GSH monoethyl ester (GSH–EE) attenuated neuropathological features of AD in SREBP-2/APP/PS1 mice.

In addition to the proteolytic processing by secretases, APP and its corresponding C-terminal fragments are also metabolized by lysosomal proteases. SORLA/SORL1 is a unique neuronal sorting receptor for APP that has been causally implicated in sporadic and autosomal dominant familial AD. Brain concentrations of SORLA are inversely correlated with Aβ in mouse models and AD patients. Indeed, transgenic mice overexpressing SORLA exhibit decreased Aβ concentrations in brain (Caglayan et al., 2014). Mechanistically, Aβ binds to the amino-terminal VP10P domain of SORLA and this binding is impaired by a familial AD mutation in SORL1. Moreover, sphingosine-1-phosphate (S1P) accumulation by S1P lyase deficiency has recently been shown to impair lysosomal APP metabolism, resulting in increased Aβ accumulation (Karaca et al., 2014). The intracellular accumulation of S1P interferes with the maturation of cathepsin D and degradation of Lamp2, suggesting a general impairment of lysosomal function and autophagy. As sphingolipids have strong affinity to bind cholesterol (Slotte, 1999; Ridgway, 2000), it is conceivable that increased lysosomal cholesterol may contribute to impaired lysosomal Aβ degradation in the S1P lyase knockout mice. However, this aspect remains to be investigated, raising the question of whether lysosomal cholesterol plays a role in lysosomal Aβ degradation and hence has any relevance in AD. Quite intriguingly, recent findings have reported increased expression of the lysosomal cholesterol transporter Niemann–Pick type C disease 1 (NPC1) in AD (Kagedal et al., 2010). NPC is an endolysosomal

protein essential for the intracellular regulation of cholesterol and its mutation and loss-of-function elicits the lysosomal storage disease NPC disease, characterized by the accumulation of lysosomal cholesterol and sphingolipids. NPC1 expression was described to be upregulated at both mRNA and protein levels in the hippocampus and frontal cortex of AD patients compared to controls subjects. However, no difference in NPC1 expression was detected in the cerebellum, a brain region that is relatively spared in AD. Moreover, murine NPC1 mRNA levels increased in the hippocampus of 12-month-old APP/PS1 mice compared to wild-type mice. These findings strongly suggest the lack of lysosomal cholesterol accumulation in AD, and imply that lysosomal impairment and subsequent contribution to decreased Aβ degradation in AD may occur through mechanisms independent of cholesterol accumulation in lysosomes. Although several similarities exist between NPC disease and AD, including altered intracelular cholesterol homeostasis, changes in the lysosomal function, neurofibrillary tangles, and increased Aβ generation and neurodegeneration, the likely common nexus between these diseases is mitochondrial cholesterol loading, rather than lysosomal cholesterol accumulation, as reported both in AD and NPC disease (Yu et al., 2005; Colell et al., 2009; Fernandez et al., 2009b). Thus, targeting mitochondrial cholesterol may be of relevance not only for AD but also for other neurodegenerative and lysosomal storage diseases, including NPC.

CONCLUSION AND FUTURE APPROACHES

Mitochondria play an essential role in providing the energy needed for multiple signaling cascades and cellular functions. The consumption of molecular oxygen in the respiratory chain not only is the driving force for the ATP synthesis required for cell viability, but also the source of ROS that target mitochondrial and extramitochondrial targets. As described above, mitochondrial oxidative stress and the mGSH depletion are central events of many pathological conditions. However, a challenge to counteract mitochondrial oxidative stress is to recover mGSH pool when GSH transport is defective due to alterations in membrane dynamics triggered by increased mitochondrial cholesterol accumulation. In addition to the ability of mitochondrial-permeable GSH-EE to directly increase mGSH levels bypassing the mitochondrial transport defect, it has been recently described additional strategies that supply mitochondria with GSH, including parental molecules that generate GSH once inside the mitochondrial matrix. This approach has been recently illustrated with the use of *S*-D-lactoylglutathione (Armeni et al., 2014). This compound is an intermediate of the glyoxalase system, which is hydrolyzed in the mitochondrial matrix yielding lactate and GSH; hence showing the ability to replenish mGSH resulting in recovery of mitochondrial function and antioxidant defense. Unlike these permeable GSH prodrugs that directly boost mGSH, strategies aimed to increase cytosol GSH (e.g., NAC) may not be an optimal approach for boosting mGSH and therefore for treatment of SH or AD, as it would result in mainly increasing cytosol GSH without replenishing mGSH levels. Another strategy to combat ROS generation would be the supply of antioxidants that are targeted selectively to mitochondria. Since lipophilic cations accumulate in mitochondria, the covalent attachment of a neutral bioactive compound to a lipophilic cation should lead to its selective delivery to mitochondria. In this regard, alkyl-triphenylphosphonium (TPP) cations are excellent tools for the delivery of compounds to mitochondria as they preferentially accumulate quite efficiently within mitochondria in cells, making it possible to deliver a wide range of mitochondria-targeted lipophilic TPP-labeled cations (Ross et al., 2005; Sheu et al., 2006). This approach has been exploited recently in several contexts by the development of a series of cationic antioxidants targeted to mitochondria, including derivatives of the endogenous antioxidants ubiquinol (MitoQ), alpha-tocopherol (MitoVit E), and of the synthetic spin trap PBN (MitoPBN; Ross et al., 2005). These compounds have been found to block oxidative damage in isolated mitochondria and cells more effectively than untargeted antioxidant analogs due to their concentration within mitochondria. More importantly, oral administration of these compounds leads to their accumulation in the brain, heart, muscle and liver mitochondria (Ross et al., 2005). In fact, MitoQ has been used in a range of *in vivo* studies, in rats and mice, and in two phase II human trials demonstrating that it can be safely delivered to patients with promising results, lending further support that mitochondria-targeted antioxidants may be applicable to a wide range of human pathologies that involve mitochondrial oxidative damage (Smith and Murphy, 2010).

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Glutathione: new roles in redox signaling for an old antioxidant

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The physiological roles played by the tripeptide glutathione have greatly advanced over the past decades superimposing the research on free radicals, oxidative stress and, more recently, redox signaling. In particular, GSH is involved in nutrient metabolism, antioxidant defense, and regulation of cellular metabolic functions ranging from gene expression, DNA and protein synthesis to signal transduction, cell proliferation and apoptosis. This review will be focused on the role of GSH in cell signaling by analysing the more recent advancements about its capability to modulate nitroxidative stress, autophagy, and viral infection.

Keywords: nitric oxide, viral infection, autophagy, redox signal, reactive oxygen species

INTRODUCTION

γ-L-glutamyl-L-cysteinyl-glycine chiefly known as glutathione (GSH) is required for several cell processes interconnected with alterations in the maintenance and regulation of the thiol-redox status, due to its capability to exist in different redox specie (Forman et al., 2009). Under physiological conditions the reduced GSH is the major form with its concentration from 10 to 100-folds higher than the oxidized species (oxidized GSH, GSSG and mixed disulphide, GSSR). GSSG is predominantly produced by the catalysis of GSH peroxidase (GPX) as well as from the direct reactions of GSH with electrophilic compounds, e.g., radical species. The production of GSSR, instead, requires a "reactive" cysteinyl residue, which at physiological pH is present in the thiolate form. These residues, under oxidative stress, are prone to oxidation in sulfenic acid, which efficiently reacts with GSH leading to a glutathionylated-cysteine derivative (GSSR). Both GSSG and GSSR can be catalytically reduced back to GSH by the NADPH-dependent GSH reductase and thioredoxin (Trx)/glutaredoxin (Grx) system, respectively. Additionally, the non-enzymatic interconversion between GSSG and GSSR can occur. Therefore, the ratio between reduced and oxidized forms of GSH is an important indicator of the redox environment and, at the same time, contributes to the accomplishment of the molecular mechanisms underlying cell proliferation, differentiation or death in the form of apoptosis (Dickinson and Forman, 2002). Moreover, it is now well established that the reversible formation of mixed disulfide GSSR through protein-*S*-glutathionylation is an important on/off mechanism for dynamic post-translational regulation of a variety of regulatory, structural and metabolic proteins involved in signaling and metabolic pathway in cell systems (Ghezzi, 2005, 2013). In this context, we also demonstrated that the disruption of the cellular redox buffer, controlled by GSH, increases not only the oxidative stress (Filomeni et al., 2005a), but also the endogenous physiological flux of NO in neuronal cells (Aquilano et al., 2011a, 2013). Indeed, GSH decrease causes protein nitration, *S*-nitrosylation, and DNA strand breaks in neuronal cells. Such alterations were also associated with inhibition of cytochrome *c* oxidase (CcOX) activity and microtubule network disassembly, which are considered hallmarks of nitric oxide (NO) toxicity, indicating that NO, rather than the depletion of GSH per se, is the primary mediator of cell damage. These results support the hypothesis that GSH represents the most important buffer of NO toxicity in neuronal cells. Moreover, the same NO/cGMP signaling was effective in survival cell response to GSH depletion in skeletal muscle cells (Aquilano et al., 2013).

On the basis of these knowledge, it is not surprising that alterations in GSH homeostasis have been implicated in the etiology and/or progression of many human diseases (Ballatori et al., 2009). In fact, a decrement of GSH levels contributes to oxidative stress associated with aging and many pathological states, including neurodegeneration, inflammation, and infections. However, given the many roles played by GSH, it has been difficult to ascribe causal relationships between changes in GSH levels or redox state and development of disease (Liu et al., 2004).

In this review we briefly summarize the mechanisms of GSH synthesis and homeostasis, while we will focus on the strict connection between GSH levels and oxidative/nitrosative stress and on the downstream redox changes that modulate signaling pathways involved in viral infection and autophagy (ATG).

GSH HOMEOSTASIS AND SYNTHESIS

Eukaryotic cells have distinct reservoirs of GSH: the majority of GSH (almost 90%) is in the cytosol, which also represents the main place for its synthesis; from cytosol, GSH is distributed

into organelles such as mitochondria, nucleus and endoplasmic reticulum (Forman et al., 2009; Lu, 2013). In these districts GSH is predominantly in the reduced form except for endoplasmic reticulum where the oxidized form is mainly present being necessary for the correct folding and secretory pathway of proteins (Hwang et al., 1992).

GSH is the most important hydrophilic antioxidant that protects cells against exogenous and endogenous toxins, including reactive oxygen (ROS) and nitrogen (RNS) species (Rauhala et al., 2005; Jozefczak et al., 2012). Among such species the radical forms are removed *via* non-enzymatic reduction with GSH, whereas the elimination of hydroperoxides requires enzymatic catalysis by GPX and catalase. The resulting oxidized form of GSH (GSSG), characterized by a disulfide bond between two molecules of GSH is efficiently reduced back to GSH by the NADPH-dependent catalysis of the flavoenzyme GSH reductase. Indeed, the GSH and GSH-related enzymatic systems are efficient tools that cells have exploited in detoxification and, at the same time, represent the most ancient notice on the physiological role played by the tripeptide. GSH in fact is both a nucleophile and a reductant, and therefore can react with electrophilic or oxidizing species rendering the former molecules more soluble and excretable, and the latter unable to interact with more critical cellular constituents such as lipids, nucleic acids and proteins. Conjugation of GSH with electrophilic compounds is mainly mediated by the glutathione-*S*-transferases (GSTs), a super family of Phase II detoxification enzymes.

GSH is synthesized *in vivo,* by the consecutive action of two ATP-dependent enzymes, from the precursor amino acids cysteine, glutamate and glycine. The first enzyme, glutamate–cysteine ligase (GCL) formerly called γ-glutamylcysteine synthase (GCS) is the rate-limiting enzyme. GCL is a heterodimer that can be dissociated under non-denaturing conditions into a modulatory or light subunit (GCLM), and a catalytic or heavy subunit (GCLC; Lu, 2013). GCL forms an unusual peptide bond between the γ-carboxyl of glutamate and the amino group of cysteine using the energy provided by the hydrolysis of ATP (Lu, 2013). GCL belongs to the class of proteins that are sensitive to oxidative stress, and its expression is mainly under the regulation of the Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), a transcription factor that regulates a wide array of antioxidant responsive element-driven genes in various cell types (Baldelli et al., 2013). The second enzyme required for *the novo* GSH biosynthesis is glutathione synthase (GS). The human GS enzyme is a homodimer of subunits containing 474 amino acid residues, encoded by a single-copy gene (Gali and Board, 1997). It catalyzes the addition of glycine to γ-glutamylcysteine created by GCL to form GSH, γ-L-glutamyl-L-cysteinyl-glycine, a reaction again driven by the hydrolysis of ATP. Finally, the chemical structure of GSH provides peculiar characteristics ranging from un-susceptibility to proteolysis to redox thiols catalysis. The overall rate of GSH synthesis is controlled by several factors including: (i) availability of the substrate mainly L-cysteine (Anderson and Meister, 1983); (ii) amount and relative ratio between the two subunits of GCL (Chen et al., 2005); (iii) extent of feedback inhibition of GCL by GSH (Taylor et al., 1996). Additionally, in some cases, the provision of ATP for GSH synthesis could represent another limiting factor. Even if virtually, all cell types synthesize GSH, the main source of the tripeptide is liver where the bulk of cysteine, the rate limiting amino acid, derived from diet is metabolized. After its synthesis, GSH is delivered to some intracellular compartments, including mitochondria, endoplasmic reticulum, nucleus, and to the extracellular space (e.g., blood plasma and bile) for utilization by other cells and tissues (Forman et al., 2009).

In contrast to GSH synthesis, which occurs intracellularly, GSH degradation occurs exclusively in the extracellular space, and in particular, on the surface of cells that express the enzyme γ-glutamyl transpeptidase (also called γ-glutamyl transferase, GGT; Ballatori et al., 2009; Baudouin-Cornu et al., 2012). The GGT is the only enzyme that can initiate catabolism of GSH and GSH-adducts (e.g., GSSG, glutathione *S*-conjugates, and glutathione complexes). GGT is an heterodimeric glycoprotein located on the external plasma membrane of specific cells present in kidney tubules, biliary epithelium and brain capillaries where it hydrolyses GSH into glutamic acid and cysteinyl-glycine; this dipeptide is further hydrolysed by cell surface dipeptidases and the resulting amino acids taken up by cells for regeneration of intracellular GSH (Pompella et al., 2007). The intra- and extracellular GSH levels are determined by the balance between its production, consumption, and transportation. Due to important physiological functions of GSH, these processes are tightly regulated. The activities of the enzymes involved in GSH metabolism are controlled at transcriptional, translational, and post-translational levels.

GSH AND OXIDATIVE STRESS

The investigation on the field related to production of ROS/RNS during metabolic processes, even under physiological conditions, unlocks another branch of research focusing on the role of GSH ranging from antioxidant/radical scavenger to redox signaling modulator. GSH effectively scavenges free radicals and other ROS and RNS (e.g., hydroxyl radical, lipid peroxyl radical, superoxide anion, and hydrogen peroxide) directly and indirectly through enzymatic reactions. The chemical structure of GSH determines its functions, and its broad distribution among all living organisms reflects its important biological role (Johnson et al., 2012). In particular, it has long been established that the thiol moiety of GSH is important in its antioxidant function in the direct scavenge of radical species. Indeed, the one-electron reduction with radicals is not chemically favorable, because it would generate the unstable thiyl radical GS. However, the reaction is kinetically driven in the forward direction by the removal of GS through the following reactions with thiolate anion (GS^-) and then with oxygen. The first reaction leads to the generation of $GSSG^-$, which in the presence of O_2, generates GSSG, and superoxide ($O_2^{\cdot -}$). Ultimately, the radical chain reactions will be blocked by the antioxidant enzymes superoxide dismutase (SOD) in association with catalase or GPX that determines the complete free radicals scavenging (Winterbourn, 1993). Indeed, we demonstrated that GSH finely compensates the decline of SOD1 activity in: (i) cells expressing less active SOD1 mutant found in familial amyotrophic lateral sclerosis (Ciriolo et al., 2001); (ii) cells in which SOD1 is down-regulated by RNA interference (Aquilano

et al., 2006; Vigilanza et al., 2008). In particular, the initial burst of superoxide that cannot be eliminated efficiently due to the inactivity of SOD1 is promptly buffered by the induction of GSH synthesis resulting in protection against oxidative stress and cell death.

On the other hand, GSH does not react directly non-enzymatically with hydroperoxides. In fact, its role as a co-substrate for the selenium-dependent GPX has been recognized as the most important mechanism for reduction of H_2O_2 and lipid hydroperoxides. Moreover, more recently a family of proteins called peroxiredoxins has been recognized as catalyzing the reduction of H_2O_2 by GSH and/or other thiols, but with cysteine in its thiolate form, in their active sites rather than selenium-cysteine (Dickinson and Forman, 2002). GSH is also involved as an antioxidant in the detoxification of products deriving from ROS-promoted oxidation of lipids such as malonyl dialdehyde and 4-hydroxy-2-nonenal, and probably many other products of ROS interaction with cellular components (Comporti, 1987). The thiyl radicals formed from these reactions can also combine with different molecules, as well as with other thiyl radicals leading to the formation of GSSG in the latter instance. GSH forms also conjugates with a great variety of electrophilic compounds, when the electrophile is very reactive, or more often through the action of GST (Eaton and Bammler, 1999; Strange et al., 2000). Many other toxic metabolites are produced as side-products of the normal cellular metabolism and some of them can be also capable to react with GSH. For example, methylglyoxal, an enzymatic, and non-enzymatic product deriving from the glycolytic pathway (Martins et al., 2001; Inagi et al., 2010), is capable to interact with any molecules containing free amino groups such as amino acids, nucleotide bases, and cysteine residues in proteins (Li et al., 2008). Methylglyoxal and other α-dicarbonyls are also involved in ROS generation. GSH acts as a cofactor in the system of methylglyoxal elimination, which consists of two enzymes called glyoxalases (Yadav et al., 2008; Inagi et al., 2010). Other functions of GSH include: (i) maintaining the essential thiol status of cysteine residues on proteins; (ii) storage of cysteine reserves; (iii) involvement in the metabolism of estrogens, leukotrienes, and prostaglandins; (iv) participation in the production of deoxyribonucleotides; (v) participation in the maturation of iron–sulfur cluster in proteins; (vi) signal transduction from the environment to cellular transcription machinery (Dickinson and Forman, 2002).

GSH AS MAIN REGULATOR OF CELLULAR REDOX STATUS AND REDOX SIGNAL TRANSDUCTION

The glutathione redox couple GSH/GSSG has a great importance in the cells and together with other redox-active couples, including NADPH/$NADP^+$, Trx-SH/Trx-SS regulates and maintains the appropriate cellular redox status. The estimated *in vivo* redox potential for the GSH/GSSG couple ranges from −260 mV to –150 mV depending on the conditions (Jones, 2002). Thus, changes in the GSH/GSSG ratio are fundamental in the fine-tuning of signal transduction, even under mild oxidative stress that underlies physiological events such as cell cycle regulation and other cellular processes (Schafer and Buettner, 2001). Conditions characterized by increased ROS levels may require not only enhanced GSH action to maintain redox status, but also augmented energy supply and precursors to replace/enhance GSH content and/or transport it to the places where it is needed. However, when oxidative stress becomes prolonged and cellular systems are no more able to counteract the oxidative-mediated insults, the amount of free GSH decreases leading to irreversible cell degeneration and death.

Later on, additional roles for the antioxidant function of GSH have emerged that are strictly related to signal transduction: (i) the interaction of the tripeptide with NO or with RNS (see next section); (ii) the involvement of GSH in the process of protein *S*-glutathionylation. Protein *S*-glutathionylation is an important post-translation modification, providing protection of protein cysteines from irreversible oxidation and, at the same time, serving to transduce a redox signal by changing structure/function of the target protein. The process is observed either under massive increase of radical species or under physiological ROS flux. The process of *S*-glutathionylation may proceed spontaneously by thiol-disulphide exchange. However, for the majority of proteins these reactions could occur only under non-physiological GSH/GSSG ratio (i.e., 1:1). There is only the case of the nuclear transcription factor c-Jun that, due to an unusual redox potential, could react with GSSG at relatively high ratio of GSH/GSSG = 13 (Klatt et al., 1999). Therefore, it is likely that mechanisms of protein *S*-glutathionylation within the cells involves reaction of the "critical cysteine" on protein or GSH with a corresponding oxidized derivative such as S-nitrosyl (*S*-NO), sulfenic acid (*S*-OH), thiyl radical (*S*). Protein-sulfenic and glutathione sulfenic acids result from reaction with endogenously produced ROS or RNS but usually these species are rapidly transformed in prot-SSG (GSSR) or GSSG as more stable derivatives. Examples that a sulfenic acid intermediate is formed during redox regulation are become known for c-Jun, Fos, nuclear factor 1, nuclear factor-κB (NF-κB), GAPDH, and PTPs and up to date there are 2200 experimentally verified *S*-glutathionylated peptides from 169 research articles (Chen et al., 2014). The reverse reaction, de-glutathionylation, is mediated by the enzyme Grx. The cytosolic mammalian form, Grx1, operates via a nucleophilic ping–pong mechanism and is highly specific for *S*-glutathionylated proteins with respect to other mixed disulfides. Instead Grx2, a mitochondrial isoform, exhibits de-glutathionylation activity for peptides and proteins but with a catalytic activity 10-fold lower with respect to Grx1 (Beer et al., 2004; Grek et al., 2013). Moreover, the forward reaction of *S*-glutathionylation can be catalyzed by glutathione-*S*-transferase P (GSTP). At physiological pH, GSTP binds GSH and lowers the p*Ka* of the thiol, producing a thiolate anion (GS^-) at the active site. This catalyzes the forward reaction, a specific example of which is provided by the reactivation of peroxiredoxin-6 (Prx). Indeed, it was demonstrated that heterodimerization of 1-cysPrx with GSH-saturated GSTP results in *S*-glutathionylation of the oxidized cysteine in 1-cysPrx followed by subsequent spontaneous reduction of the mixed disulfide and restoration of enzymatic activity (Manevich et al., 2004).

GSH AND NITROSATIVE STRESS

Besides protecting against ROS, GSH is implicated in counteracting RNS-mediated damage. The intracellular GSH concentration appears to be an important factor in driving susceptibility to NO and its derivatives. NO is synthesized from L-arginine by enzymes known as nitric oxide synthases (NOSs; Knowles and Moncada, 1994). There are three genetically different isoforms of NOS. They include neuronal NOS (also known as nNOS, type I, NOS-1, and NOS-I), being the isoform found in neuronal tissues; inducible NOS (also known as iNOS, type II, NOS-II, and NOS2), being the isoform which can be synthesized following induction by pro-inflammatory cytokines or endotoxin and endothelial NOS (also known as eNOS, type III, NOS-III, and NOS-3), being the isoform expressed in endothelial cells (Murphy et al., 1993; Nelson et al., 2003; Maron and Michel, 2012).

Overproduction of NO can occur in several pathological conditions (e.g., inflammation, endotoxic shock, diabetes, ischemia/reperfusion injury; Wong and Billiar, 1995; Iadecola, 1997; Szabo, 1998; Friederich et al., 2009) or upon exposure to pharmacological drugs (Scatena et al., 2010) or ionizing radiation (Azzam et al., 2012). Overall these conditions stimulate the expression of iNOS, thus producing large amounts of NO. By a variety of mechanisms, NO can react with different molecules, under ambient oxygen, and the derived products can damage the cellular macromolecules, such as lipids, DNA bases, proteins as well as thiols. A primary reaction of RNS formation is the combination of NO and superoxide ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$) with a rate constant that is larger than that for the SOD-catalyzed dismutation of $O_2^{\cdot -}$ (Jay-Gerin and Ferradini, 2000).

Following NO/$ONOO^-$ exposure, significant effects on cellular GSH metabolism are reported. NO can react with the cysteine of GSH to form GSNO that may be considered an endogenous NO reservoir, which can release it when it reacts with Cu^+ or with denitrosylating enzymes such as Trx and GSNO reductase (Sengupta and Holmgren, 2013). The same reaction can occur on protein cysteine, and the formed GSNO group functions as important intermediate in NO metabolism, a critical player in fulfilling the biolgogical function of NO in signal transduction (Chung, 2006). Besides functioning as a NO donor, GSNO can also mediate protein *S*-glutathionylation (Chiueh and Rauhala, 1999). GSNO can be also exported out of the cells via a the GSH transporter. In the extracellular milieu GSNO can undergo a transnitrosation reaction with cystine (Zeng et al., 2001) or thanks to catalytic activity of GGT generate S-nitrosocysteinylglycine that in turn can be cleaved by a membrane dipeptidase thus forming *S*-nitrosocysteine (S-NO). *S*-nitrosocysteine can enter the cells via the L-type amino-acid transporter (Hogg et al., 1997) and be reduced to release NO or transfer NO to proteins and thus mediate NO signaling. The way by which GSNO is formed has been the topic of several studies and many mechanisms have been proposed as extensively illustrated in the literature (Singh et al., 1996; Forman et al., 2004). Briefly, at typical physiological GSH concentrations and pH, the reaction between $ONOO^-$ and GSH will predominantly be a two electron oxidation process leading to GSSG formation (Quijano et al., 1997). However, one electron oxidation process involving peroxynitrous acid and/or its derivatives can also occur. Under these circumstances a thiyl radical is formed that will initiate and propagate an oxygen-dependent chain reaction, involving peroxyl radical formation that will lead to further consumption of intracellular GSH (Quijano et al., 1997). Such reactions will subtract RNS from critical cellular targets, such as cell membrane lipids and proteins belonging to the complexes of the electron transport chain, thereby explaining why GSH status appears to be so critical in modulating the susceptibility to NO.

GSH AS A PHYSIOLOGICAL MODULATOR OF NO HOMEOSTASIS

The importance of GSH in protecting the electron transport chain from $ONOO^-$ is illustrated by experiments with GSH depleted astrocytes. Under such conditions marked damage to the ETC and cell death occurs following $ONOO^-$ exposure (Barker et al., 1996). Conversely, the apparent increase in resistance of neurons to NO when co-cultured with astrocytes can be explained by considering the effect of co-culture upon the neuronal GSH concentration (Bolanos et al., 1996). Under these conditions, up-regulation of neuronal GSH is thought to occur as a result of the astrocytic release and preservation of GSH followed by cleavage, via GGT.

The effect of NO and its derivatives upon cellular GSH metabolism is also dependent on factors such as cell type, cellular environment and duration of exposure. For instance, induction of iNOS in microglia leads to a decrease in cellular GSH level, whereas, under comparable conditions, the GSH status of astrocytes remains largely unaffected (Bolanos et al., 1994; Chatterjee et al., 2000) Similarly, the GSH concentration in astrocytes does not appear to be affected by $ONOO^-$ exposure (Bolanos et al., 1995). This apparent preservation of GSH may reflect the greater activity, in astrocytes, of GCL (Makar et al., 1994). Conversely, others and our laboratory have demonstrated that NO can favor GSH synthesis via the NEFL2-mediated transcription of GCL (Baldelli et al., 2008; Cortese-Krott et al., 2009).

The role of GSH as NO scavenger is also highlighted by Atakisi et al. (2010), who show how intraperitoneal administrations of GSH in rabbits cause a lowering of NO levels in plasma (Atakisi et al., 2010), suggesting that exogenous GSH may be a valuable enhancer of the antioxidant system. On the same line of evidence, supplementation of cells of neuronal origin with a membrane permeable GSH derivative (GSH60) protects them from NO neurotoxicity (Hu et al., 2012).

To investigate the importance of correct GSH homeostasis for the neurotrophic capacity of NO, Canals et al. (2001) pre-treated primary midbrain cultures with different doses of buthionine sulfoximine (BSO) a specific inhibitor of GCL, the rate-limiting enzyme in GSH synthesis. Under these conditions, NO triggers a programmed cell death with markers of both apoptosis and necrosis characterized by an early step of free radicals production followed by a late requirement for signaling on the sGC/cGMP/PKG pathway (Canals et al., 2001). Similarly, in our laboratory we demonstrated that neuronal cells are highly vulnerable to physiological flux of NO in the absence of GSH (Aquilano et al., 2011a). In particular, our study showed that the intracellular depletion of GSH is able to induce cellular stress in NO-producing cells through a NO-dependent mechanism, such as inhibition of CcOX activity, DNA damage, and S-NO and 3-nitrotyrosine (NO_2-Tyr) protein accumulation (**Figure 1**). Moreover, NO seems

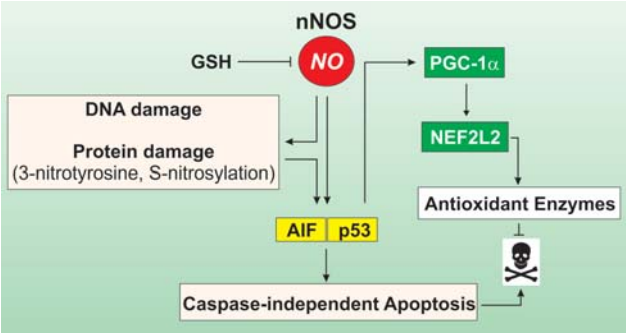

FIGURE 1 | Role of GSH in modulating cell response to NO. In cells of neuronal or muscle origin NO is produced by neuronal nitric oxide synthase (nNOS). Intracellular GSH is a crucial factor in modulating NO reactivity, as it functions as an efficient NO buffer. When GSH levels decline, NO availability is increased and may trigger DNA damage as well as protein oxidation, in terms of *S*-nitrosylation of cyteines and formation of 3-nitrotyrosine on protein residues. This leads to induction of caspase-independent apotosis via the activation of the apoptosis inducing factor (AIF). Concomitantly, p53 is activated and binds to consensus sequence of PGC-1a promoter increasing its expression. PGC-1a in turn co-activates the NEFL2-dependent expression of antioxidant genes, thus limiting oxidative damage and cell death.

to be the only mediator of cell proliferation arrest through the ERK1/2-p53 signaling pathway (Aquilano et al., 2011a). We also reported that even a slight and non-toxic decrease of GSH in brain mice causes protein nitration that is reversed by inhibiting NO production (Aquilano et al., 2011b). This evidence indicates that NO imbalance and the associated nitrosative stress observed in neruodegenerative disease and during aging are likely the consequence of the progressive decline of GSH (Aquilano et al., 2011b).

Next to this evidence, in our laboratory it has been demonstrated that GSH depletion modulates the peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1α) expression and its downstream metabolic pathway in neuronal and skeletal muscle cells (**Figure 1**). This effect was abrogated by inhibiting NOSs or guanylate cyclase, implicating NO/cGMP signaling pathway in this process (Aquilano et al., 2013). In particular, we found that the moderate depletion of GSH is operative upon fasting conditions in several organs including skeletal muscle and brain and this event is causative of the augmentation of NO availability. The increased NO bioavailability and the down-stream p53-mediated induction of PGC-1α favors the expression of NFE2L2 and its antioxidant-related genes (**Figure 1**). Thus, in these circumstances GSH, by subtracting biological active NO, could impede the activation of a more effective antioxidant response, which is involved in the increased lifespan.

GSH TIPS THE SCALES BETWEEN SURVIVAL AND CELL DEATH

The ROS-mediated intrinsic pathway of apoptosis disclosed that the intracellular amount of GSH could determine the capability of the cell to undergo apoptosis. This process represents the summary of the GSH-related aspects with regards to: (i) changes in cellular GSH redox homeostasis through decreased GSH/GSSG ratio, due to either GSH oxidation or active GSH export in relation to the initiation or execution of the apoptotic cascade; (ii) evidence for *S*-glutathionylation in protein modulation and apoptotic initiation. The story about GSH and apoptosis starts around the 1990s with the evidence of an active GSH extrusion from the cell undergoing apoptosis (Ghibelli et al., 1995; van den Dobbelsteen et al., 1996) and with the subsequent conflicting notion that chemical depletion of GSH was not, however, sufficient to induce the apoptotic process (Ghibelli et al., 1999; Filomeni et al., 2005b). What is worth to summarize here is the fact that several pro-apoptotic stimuli induce an early extrusion of GSH from cells leading to a widespread mitochondrial damage and to cytochrome *c* release into the cytosol, the starting condition for apoptotic intrinsic pathway commitment. This event was, however, not necessarily preceded by an oxidative burst and later on we demonstrated that GSH release was the result of an active process that the cells carried out through specific carriers in order to efficiently execute the apoptotic program (Ghibelli et al., 1998). The picture illustrated was much more complex, because in several experimental systems, cell viability was not affected when GSH depletion was obtained chemically by inhibition of its synthesis. It was, therefore, concluded that GSH content is important for cell response to detrimental insults but not the principal event underlying the mitochondrial route of apoptosis. Finally, GSH depletion was designated as *necessary* to induce the redox unbalance characteristic of the induction phase of the mitochondrial pathway but not sufficient *per se* to assure the execution phase of apoptosis.

Successively the scenario was enriched by several molecular players that belong to redox-sensitive factors such as the Bcl-2 family (Hockenbery et al., 1993; Kowaltowski and Fiskum, 2005; Czabotar et al., 2014; Moldoveanu et al., 2014), p53 (Sun et al., 2003; Wang and Gu, 2014), c-Jun/AP-1 (Marshall et al., 2000), NF-κB (Pineda-Molina et al., 2001), heat shock factor 1 (HSF1; Jacquier-Sarlin and Polla, 1996; Anckar and Sistonen, 2011), and NE2FL2 (Itoh et al., 2004; Kansanen et al., 2013) together with the upstream mediators (dead receptors; Sullivan et al., 2000) of phosphorylative cascade, MAPKs (Filomeni et al., 2005c; Filomeni and Ciriolo, 2006), and downstream targets (mitochondrial and cytoskeleton proteins). Apoptosis began to be considered a much more fine-regulated process, being achieved only when the intracellular levels of GSH were affected in a well defined order, depending on the GSH-related redox pattern of the cell (Circu and Aw, 2012). The most intriguing and at the same time explanatory example comes from cancerous cells treated with ROS or with thiol-oxidizing agents. In particular, adenocarcinoma gastric (AGS) cells activated the mitochondrial pathway of apoptosis upon treatment with thiol-oxidizing agents, such as diamide, while they were resistant to hydrogen peroxide. Both responses correlated with GSH redox changes, with diamide increasing GSSG, and hydrogen peroxide inducing protein-GSH mixed disulfides (GSSR). We demonstrated that p53 was activated in response to diamide treatment by the oxidative induction of the Trx1/p38(MAPK) signaling pathway, which finally resulted in apoptosis. On the contrary, resistance to ROS was achieved by means of the redox activation of NE2FL2 (Filomeni et al., 2012). Neuroblastoma SH-SY5Y had a complete opposite

behavior, being sensitive to hydrogen peroxide, but resistant to diamide. In this cells, the apoptotic pathway relied upon the same Trx1/p38MAPK/p53 signaling axis and cell survival to diamide relied upon redox activation of NE2FL2, in a way independent of Keap1 oxidation, but responsive to ERK1/2 activation (Filomeni et al., 2012). It was demonstrated that the molecular determinant(s) unifying these phenomena, was related to GSH and GSH-sensitive molecular factors. Indeed, SH-SY5Y cells showed high GSH levels but exhibited very low GPx activity, whereas AGS cells had low GSH content but several isoforms of the GPx enzyme.

The outstanding progress accomplished in the last years allows to assert that, even though the pathways implicated in the induction/execution of apoptosis have remained largely unaltered, many new redox-dependent processes have been added and we suggest the reading of several reviews on this subject (Anathy et al., 2012; Circu and Aw, 2012).

From the first evidence regarding the involvement of GSH in apoptosis, up to date many improvements have been made, and growing results, which attempted to dissect the mechanisms linking GSH to cellular redox-dependent processes, were added. The scenario depicted, clearly indicates that GSH is a *weighty protagonist* of the huge network governing the decision between life and death, through the modulation of cellular redox state. Indeed, GSH, Trx, Grx, Prx (together with the enzymes catalyzing their reduction), and the enzymatic antioxidant defense, as a whole, concur to modulate either ROS concentration, or the availability of cellular thiols, thus regulating the function of a large number of proteins implicated in the induction and/or execution of redox-sensitive pathways. Among such pathways ATG seems to be also included (Filomeni et al., 2010).

Autophagy is a degradation pathway essential for maintaining cellular homeostasis during different stressful conditions. Three distinct types of ATG are so far identified: (i) macro-ATG; (ii) micro-ATG, and (iii) chaperone-mediated ATG. Macro-ATG consists in the delivery of cytoplasmic cargo to the lysosome through the intermediary of a double membrane bound vesicle, the autophagosome, which fuses with the lysosome to form an autolysosome (Zhang, 2013). This process also contributes to the removal of damaged organelles, such as mitochondria and ER, and misfolded or aggregate-prone proteins (Mizushima, 2011). Moreover, as component of cellular integrated stress responses, ATG balances source of energy at critical times; e.g., under nutrient starvation, being able to provide substrates for ATP production and macromolecule synthesis (Zhang, 2013). Several ATG-related proteins were identified and their role in the molecular mechanisms of ATG highlighted. Since several ATGs and lysosomal cathepsin proteases depend on cysteines for their activities they can be the sensors or transducers of changes in the intracellular redox state through mechanisms involving redox signaling.

Accumulating data point to an essential role for ROS as mediators in the activation of ATG under several stimuli (starvation, pathogens, death receptors), even though the nature of this involvement remains still elusive. However, under starvation cells increase production of mitochondrial-derived hydrogen peroxide, which has been shown to be partly dependent on the activation of class III PI3K (Scherz-Shouval and Elazar, 2007). N-acetylcysteine, an efficient thiol antioxidant and GSH precursor, was able to inhibit ATG by impeding the lipidation process of LC3 mediated by ATG4, the activity of which is modulated by hydrogen peroxide (Scherz-Shouval et al., 2007). Successively, it was shown that TIGAR, a protein involved in the inhibition of the glycolytic pathway, could limit starvation-induced ATG by means of its ability to indirectly suppress ROS production (Bensaad et al., 2009). In fact, TIGAR redirects glucose-6-P toward the pentose phosphate pathway helping to lower ROS by the NADPH-dependent antioxidant systems. However, despite the plethora of observations regarding ROS and ATG, the only direct observation on the involvement of signal transduction mechanisms regulating self-digestion upon thiol/disulfide changes was evidenced in yeasts (Deffieu et al., 2009). It was demonstrated that GSH level affects the selective elimination of mitochondria (mitophagy) in *S. cerevisiae*. Also in this case the thiol antioxidant *N*-acetyl-cysteine prevented the delivery of mitochondria to the digestive vacuoles. The inhibition was specific for mitophagy because neither macro- nor micro-ATG was altered. Moreover, chemical or genetic manipulation of GSH pool stimulated mitophagy but not general ATG. These data, while revealing that mitophagy can be regulated independently of general ATG, at the same time, outlined a role for cellular redox status in its commitment.

These observations allowed postulating that intracellular redox state of the thiol pool, which strongly depends on GSH level, can drive autophagic response at multiple levels (Filomeni et al., 2010). We demonstrated that, although ROS are necessary for the initiation phase of ATG, the modulation of thiols are fundamental for the progression of starvation-induced ATG (Desideri et al., 2012; **Figure 2**). Indeed, upon nutrient deprivation GSH was efficiently extruded from cells. This process, along with GCL inhibition and the formation of mixed disulfides between GSH and protein sulfhydryl, concurred to a steady-state decrement of free GSH concentration and, consequently, to an intracellular redox state shift toward more oxidizing conditions, which could be required for avoiding fast reduction of oxidized cysteines allowing the upstream ROS-induced autophagic stimulus to be propagated. From these results it is emerging that, under increased ROS flux, the cell necessitates to efficiently extrude GSH in order to propagate the redox stimulus. Moreover, ATG and apoptosis induction had common starting condition with ATG underlying first cell response (survival) before commitment to apoptosis. More recently, we also evidenced that starvation induced $p38^{MAPK}$ activation, which by reprogramming glucose metabolism by the pentose phosphate pathway sustains NADPH production. This process was fundamental in order to buffer ROS flux and a consequent detrimental over increase of self-digestion (Desideri et al., 2014; **Figure 2**). Overall the above reported evidence on the involvement of GSH and the redox environment are only some issues that deserve to be further investigated to comprehend the several aspects characterizing the possible crosstalk between redox reactions and modulation of ATG. Indeed, redox modulation of ATG could impinge several fields interconnected with bioenergetics and metabolic adaptations to different stimuli. In this context, the peptidase, DJ-1 could have a prominent role because it possess

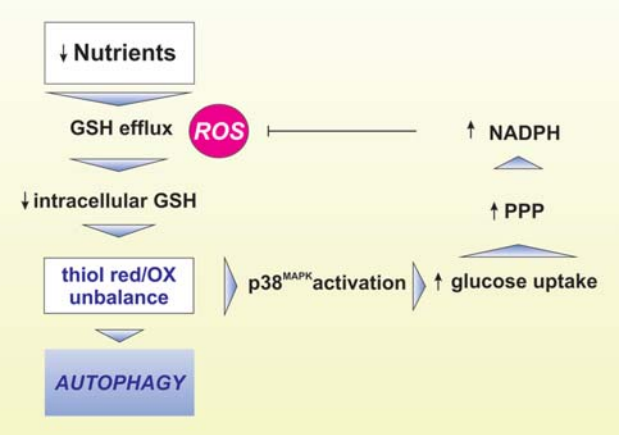

FIGURE 2 | Role of GSH in the modulation of ATG. Lack of nutrients leads to increased reactive oxygen species (ROS) production concomitant to intracellular GSH efflux through the cell membrane. These events result in thiol redox unbalance leading to activation of crucial proteins involved in ATG induction and execution. Under nutrient restriction, the phoshorylative signaling cascade governed by $p38^{MAPK}$ favors glucose uptake leading to the increase of NADPH production via the enhancement of pentose phosphate pathway (PPP). NADPH in turn contributes to buffer higher ROS-mediated oxidative damage.

reactive cysteines and is a modulator of mitochondrial dynamics and mitophagy (Thomas et al., 2011). Moreover, recently it has been reported that the pathway governed by NE2FL2 is not only regulated by the proteasome but also by ATG (Jain et al., 2013). The p62/SQSTM1 (sequestosome 1) protein, which acts as a cargo receptor for autophagic degradation of ubiquitinated proteins, is up-regulated by various stressors. The inhibitory subunit of NE2FL2, Keap1, is a cysteine-rich protein that serves as a redox sensor and can bind to p62 and be degraded by autophagic activity. In response to oxidants, cysteine modification on Keap1 releases NE2FL2, which is then stabilized and transported to the nucleus where it activates transcription of antioxidant genes, including proteasomal PA28αβ. This results in increased proteasomal degradation of oxidatively damaged proteins as well as p62, thereby facilitating ATG-lysosomal degradation of proteins and dysfunctional organelles, such as the mitochondria (Levonen et al., 2014).

Apoptosis and ATG are processes altered in cancer cells that show critical metabolic transformations associated with enhanced cellular stress. Adaptation to such conditions is characteristic of cancer cells survival and the underlying processes of resistance to apoptosis and/or efficient ATG induction. Therefore, antioxidant therapy was acclaimed as a valuable tool to selectively kill cancer cells. Results obtained from randomized clinical trials, however, were inconsistent and in some circumstances have indicated that antioxidants increase cancer risk (Klein et al., 2011; Watson, 2013). Moreover, it was reported that supplementing the diet with the antioxidants *N*-acetylcysteine and vitamin E markedly increases tumor progression and reduces survival in mouse models of B-RAF- and K-RAS-induced lung cancer, by reduction of ROS, DNA damage, and p53 expression (Sayin et al., 2014). On the contrary, the use of small molecules derived from diet that alter the levels of ROS, such as diallyl disulfide, polyphenols, isothiocyanates, and terpenoids has been suggested for the treatment of cancer by promoting ROS generation and GSH depletion in cancer cells (Filomeni et al., 2003; Shankar et al., 2006; Trachootham et al., 2006; Yue et al., 2006; Aquilano et al., 2010). Interestingly, depletion of mitochondrial GSH has also been associated with apoptosis or ATG induced by chemotherapeutic drugs (Samudio et al., 2005; Chen et al., 2011).

From these data we can reasoned that by selectively targeting GSH content of cancer cells we can induce apoptosis either directly or by combining therapies with redox antineoplastic agents.

GSH AND VIRAL INFECTION

In this section we plan to discuss the role of the tripeptide GSH in the process dealing with modulation of viral infection from the point of view of intracellular redox shift necessary for the efficient replication cycle. We are aware that several other microbial organisms alter the GSH content of cell and infected organism and very recently it was published a review on GSH and infection (Morris et al., 2013) that we suggest for readers who are interested in more wide knowledge on this topic. The choice is also dictated by the limit in length imposes for this review.

One of the first evidence involving GSH homeostasis alteration in viral infection was a paper published by Buhl et al. (1989). In this study, a 30% decrease in venous plasma and 60% decrease in epithelial lung fluid of asymptomatic HIV-seropositive subject were registered. Since GSH is a powerful stimulator of immune function, it was hypothesized that its loss could be critical for the immunodeficiency onset during HIV infection. Successively, oxidative phenomena and GSH were involved in the induction of HIV expression. Indeed, the supplementation of GSH precursors N-acetyl cysteine or GSH esther to HIV-infected cultured monocytes was found to efficiently inhibit HIV expression (Kalebic et al., 1991). Then, other reports suggested that impaired antioxidant defense, and in particular altered GSH metabolism, play important roles in HIV infection. Indeed, HIV-infected cells, besides having decreased level of intracellular GSH, display increased generation of ROS along with high rate of GSSG formation (Droge et al., 1994). T cells isolated from HIV-infected patients had lower cysteine and GSH contents (de Quay et al., 1992; Staal et al., 1992). Moreover, GSH levels were found decreased in peripheral blood mononuclear cells, and monocytes of HIV infected individuals (Buhl et al., 1989; Morris et al., 2013). Notably clinical studies carried out in 1997 directly demonstrated that low GSH levels in CD4 T cells predict poor survival in otherwise indistinguishable HIV-infected subjects (Herzenberg et al., 1997). Importantly, the oral administration of *N*-acetyl cysteine restored GSH blood levels, suggesting the use of this drug for HIV treatment and improving survival. Overall these findings point that the reducing milieu created by GSH inhibits the triggering of oxidation-dependent signal transduction pathways associated with HIV expression. Actually, decreased GSH levels are known to activate NF-κB transcription factor, which has been shown to bind and activate genes controlled by the HIV long terminal repeat (Staal, 1998), and thus may affect viral replication.

Successively, GSH was also found to be a powerful inhibitor of other viruses including herpes simplex virus type 1 (HSV-1), Sendai and influenza. We have demonstrated that during viral infections a decrease of intracellular GSH is operative that varies in intensity, duration and mechanism of induction depending on the type of virus and the infected host cell (**Figure 3**; Garaci et al., 1992, 1997; Palamara et al., 1995, 1996; Ciriolo et al., 1997). We demonstrated that GSH supplementation directly inhibits the production of mature viral particles by interfering with virus envelope glycoproteins. Indeed, administration of GSH permeable analog GSH-C4 blocks the replication of several influenza virus strains (Sgarbanti et al., 2011). All viral glycoproteins share the characteristic of their assembly into oligomers that necessitates the formation of a disulfide bond and this is inhibited by the reducing action of GSH. For instance, the influenza HA glycoprotein is a homotrimer in which each monomer consists in two-disulfide linked subunits. Cells harboring high levels of GSH efficiently counteract the glycoprotein assembly and coherently the intracellular depletion of GSH, by its synthesis inhibitor BSO, favors influenza virus replication (Nencioni et al., 2003). To set a more suitable pro-oxidant milieu critical for maturation of HA, influenza virus also impinges GSH loss (Nencioni et al., 2003; **Figure 3**). Moreover, we also found that a strict relationship exists between the level of the GSH intracellular content, anti-apoptotic protein Bcl-2 and the intensity of influenza virus replication. In particular, we substantiated that high levels of Bcl-2 leads to increased amount of GSH and finally impair virus infection (Nencioni et al., 2003). However, Bcl-2 and GSH interfere with two independent routes of viral replication cycle. Indeed, while GSH negatively impact with the expression of late viral proteins (in particular hemagglutinin and matrix), Bcl-2 impairs nuclear-cytoplasmic translocation of viral ribonucleoproteins (vRNPs). In this regard, we have successively documented that the formation of a Bcl-2/p38MAPK heterocomplex into the cytoplasm is the genuine responsible for the inhibition of vRNPs nuclear export. More in dept, Bcl-2 impedes p38MAPK translocation into the nucleus, wherein it should be responsible for phosphorylation-dependent NP export from the nucleus. As result, the blockage of NP into the nucleus dramatically affects viral packaging and final maturation (Nencioni et al., 2009).

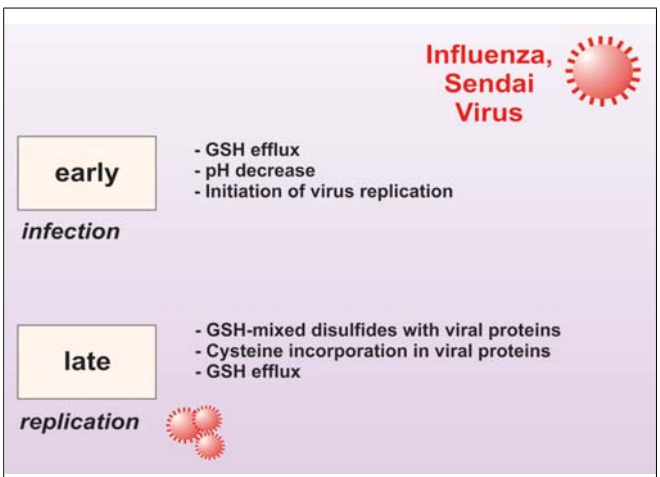

FIGURE 3 | Modulation of intracellular GSH level during Sendai and Influenza virus infection. In the early phase of infection, host cell membrane perturbation causes decrease of intracellular pH and GSH efflux allowing virus cycle start. In the late phase of infection, GSH is also engaged in forming mixed disulfides with viral proteins. GSH intracellular levels are further decreased as its component cysteine is incorporated into viral proteins.

Also other authors have shown the importance of GSH for influenza virus infection and replication. In fact, Madin-Darby canine kidney cells or human small airway epithelial cells infected with influenza virus and treated with reduced GSH exhibited an inhibition of viral matrix proteins. Moreover in BALB/c mice, inclusion of GSH in the drinking water decreased viral titer in both lung and trachea homogenates after intranasal inoculation with a mouse-adapted influenza strain A/X-31 (Cai et al., 2003).

Our group demonstrated that GSH was able to inhibit also HIV expression in human macrophages at late stages and this was related to the selective decrease of specific glycoproteins, such as gp120, which are notably rich in disulfide bonds (Garaci et al., 1997).

Next to this, it has been also reported that *in vitro* infection and replication of human herpes simplex virus type 1 (HSV-1) induced a dramatic decrease in intracellular GSH. The addition of exogenous GSH was not only able to restore its intracellular levels almost up to those found in uninfected cells, but also to completely inhibit the HSV-1 replication (Palamara et al., 1995). Similarly, it has been reported that Madin-Darby canine kidney cells infected with Sendai virus rapidly lost GSH, without increase in the oxidized products. In this condition of viral infection, oxidative stress is imposed by GSH depletion, occurring in two steps and following direct virus challenge of the cell membrane, without the intervention of ROS (Ciriolo et al., 1997). Furthermore, we have demonstrated that a direct administration of high doses of GSH exerts antiviral activity and improves immune functions in a murine immunodeficiency animal model. In fact, evaluation of pro-viral DNA content showed that GSH was effective in inhibiting the infectivity of murine leukemia virus-infected mice (LP-BM5) in lymph nodes, spleen, and bone marrow. Thus, GSH reduces the pro-viral DNA load in the first period of infection, suggesting that this antioxidant may be useful for improving current antiviral therapies (Palamara et al., 1996).

Also the pathogenesis of dengue virus (DV) infection has been closely linked to the GSH concentration. In fact, Tian et al. (2010) have demonstrated that DV serotype 2 (DV2) infection resulted in a decrease in intracellular GSH level, which caused NF-κB activation and increased DV2 production (Tian et al., 2010). Supplemental GSH significantly inhibited activation of NF-κB, resulting in a decreased production of DV2 in HepG2 cells. Furthermore, high activity of NF-κB and increased production of DV2 was observed in HepG2 cells treated with BSO. In conclusion, DV2 infection could reduce host intracellular GSH concentration and benefited from this process. Supplemental GSH could inhibit viral production, indicating that GSH might be valuable in the prevention and treatment of DV2 infection (Tian et al., 2010). Additionally, it has been demonstrated that also chronic hepatitis C virus (HCV)

infection in hepatocyte cell lines led to a decreased levels of reduced GSH and a concomitant increase of oxidative stress (Abdalla et al., 2005).

The altered intracellular redox state has been envisaged as target for anti-influenza therapy, but also in this case the data arising from *in vivo* studies are inconsistent, and in some cases have indicated that antioxidants could exacerbate the disease. The most ascertain example is the use of vitamin C in the prevention and treatment of the common cold, which has been a subject of controversy for at least 70 years (Hemila and Chalker, 2013). However, Friel and Lederman (2006) published that a nutritional supplementation of antioxidants may be effective against the influenza A (H5N1) infection. The cocktail suggested included also GSH and its precursor *N*-acetylcysteine that could avoid the rapid and detrimental loss of the intracellular content of the tripeptide during infection. In this case, as also stated by the authors, the supplementation could be efficacious only if it is going to be prophylactically used prior to an H5N1 influenza infection. However, no data are available on the real efficacy of such formulation on the pathogenesis of H5N1 influenza.

CONCLUSION

Glutathione displays remarkable metabolic and regulatory versatility, which poses the tripeptide at the center stage of a multitude of cellular processes, including cell proliferation, differentiation, and death. Some of the molecular mechanisms underlying the modulation of such processes by GSH have been established and mainly linked to the modulation of cellular redox state. The most intriguing aspect that is nowadays emerging is the crosstalk between ATG and apoptosis through a link with redox signaling governed by GSH. We expect in the near future growing recognition of GSH involvement, by *S*-glutathionylation and/or by the thiols redox state shift, not only in the induction of ATG and/or apoptosis, but also in the modulation of activities of molecular/transcription factors such as NEF2L2, PGC1α, and p53 that by inducing the antioxidant defense and assuring mitochondrial homeostasis could block both processes.

Finally, GSH deficiency contributes to oxidative/nitrosative stress, a condition actually connected with the pathogenesis of many diseases, including cancer, diseases of aging, cystic fibrosis, infection and neurodegeneration. Therefore, elucidating the mechanisms through which GSH is involved in ATG and/or apoptosis will be crucial to develop advanced therapies to counteract or ameliorate such diseases.

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Glutathione depletion in survival and apoptotic pathways

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GSH EXTRUSION IN APOPTOSIS

Damaged cells opting for apoptosis actively extrude glutathione in the reduced form as an apical event in the apoptotic signaling (Ghibelli et al., 1995; Van den Dobbelsteen et al., 1996). Apoptotic GSH efflux is very rapid and, as a consequence, cells undergo redox imbalance and become susceptible to oxidative stress due to loss of ROS scavenging power (D'Alessio et al., 2003); moreover, the sudden redox disequilibrium causes the formation of inter-protein disulfides among reactive exposed cysteines, altering cell functions and signaling in a pro-apoptotic fashion (Circu and Aw, 2012).

GSH EFFLUX IN THE LIFE AND DEATH OF A CELL

Glutathione is synthesized in the cell cytosol, and acts essentially as an intracellular antioxidant. However, viable cells (mainly hepatocytes and macrophages) extrude GSH as part of their physiological functions; this provides antioxidant protection to the extracellular environment (Bachhawat et al., 2013). The passage of GSH through plasma membrane is regulated by a switch in the open/closed configuration of the transporters, and occurs according to gradient, being in fact uni-directional (i.e., export rather than import) because intracellular levels are much higher than those of body fluids. GSH transporters were originally functionally identified as belonging to the sinusoidal or canalicular type according to their position in the hepatic anatomy, and to their responsiveness to specific inhibitors (Yi et al., 1995). However, their molecular nature is still elusive (Bachhawat et al., 2013). Based on several experimental evidences, the prevalent current opinion is that GSH transporters coincide with the multi-drug resistance associated proteins (MRP) (Ballatori et al., 2009; Franco and Cidlowski, 2012), which constitute the complexes responsible for cell detoxification from xenobiotics through the export of glutathione-S conjugates produced by glutathione-S-transferase (Pompella et al., 2003). The regulation of the activity of GSH transporters is still unexplored, possibly being controlled by the differential concentration of GSH in the vicinity of the carriers in the internal vs. external face of the cell membrane, thereby relying in the zonal control of GSH levels by intracellular trafficking.

A different scenario applies to the export of the oxidized glutathione dimers (GSSG) formed during oxidative stress (and not reduced back to GSH by glutathione reductase). Even though GSSG was shown to be target of MRP (Ballatori et al., 2009), in conditions of oxidative stress GSSG exits from cells passively crossing the plasma membrane. This mechanism helps avoiding a dangerous drop in the GSH/GSSG ratio due to the accumulation of GSSG, and the consequent redox imbalance.

Glutathione loss in apoptosis has been subject of intense investigation. Most apoptotic cells become devoid of glutathione even before rupture of the plasma membrane (Ghibelli et al., 1995), which indicates an active phenomenon of extrusion. In instances of apoptosis induced by oxidative insults, a passive efflux of GSSG was observed (Esteve et al., 1999); in this case, GSH loss is a consequence of oxidative stress. In instances of apoptosis induced by non-oxidative insults, glutathione is extruded outside cells in the reduced form immediately prior to the execution of apoptosis (Ghibelli et al., 1995; Van den Dobbelsteen et al., 1996), followed by ROS production as a consequence (D'Alessio et al., 2003); in this case, GSH loss is the cause, rather than the consequence of oxidative stress.

Apoptotic GSH extrusion is prevented by inhibitors of the sinusoidal GSH transporters (Ghibelli et al., 1998), indicating that apoptotic GSH efflux follows the same route as physiological GSH export. However, while healthy cells shed only a limited fraction of their GSH, in apoptosis the efflux leads to complete GSH depletion, suggesting that the transporters remain stably in an open configuration. The events that cause this phenomenon are still unknown; we have preliminary evidence that the activation of the apoptotic protease caspase-2, but not the other caspases, is required for apoptotic GSH efflux, suggesting that a proteolytic event (the digestion of an inhibitor of the transporters?), might cause an irreversible opening of the GSH transporters (De Nicola et al., in preparation).

ROLE OF APOPTOTIC GSH EXTRUSION

GSH extrusion is a necessary step of the apoptotic signal transduction (Coppola and Ghibelli, 2000), occurring as an apical and causative event, as shown by studies demonstrating that inhibitors of GSH efflux also inhibit apoptosis (Ghibelli et al., 1998). The block of the apoptotic signaling occurs as a very early step, because even apical events such as Bax translocation are prevented. Antioxidants do not hamper apoptotic GSH efflux, but protect GSH-depleted cells from apoptosis

(Liuzzi et al., 2003), indicating that the sudden GSH drop activates a redox-sensitive target among the apical pro-apoptotic proteins. A likely target is Bax, a pro-apoptotic member of the Bcl-2 family that in apoptosis moves to mitochondria, where it is responsible for the release of cytochrome c and the consequent caspase activation and cell demise (Ghibelli and Diederich, 2010). Bax has two exposed cysteines (Suzuki et al., 2000) able to form inter-protein disulfide bridges upon oxidation (D'Alessio et al., 2005), and therefore it can be at the same time a sensor of oxidation and an effector of apoptosis. Indeed, Bax was experimentally shown to assemble in an oxidative dimer configuration, putatively forming a disulfide between cysteine 62 and cysteine 126, thereby acquiring the ability to translocate to mitochondria (D'Alessio et al., 2005) and promote cytochrome c release (Ghibelli et al., 1999).

Damage-induced apoptosis was shown to follow either redox-sensitive or -insensitive routes, characterized by GSH depletion or retention, respectively, through alternative biochemical routes that can be morphologically identified (De Nicola et al., 2006). Leukemic cells display multiple nuclear apoptotic morphologies; in particular, two types of nuclear vesiculation (budding and cleavage) are the result of two independent morphological routes, since they never interconvert and are differently modulated by damage vs. physiological apoptogenic agents (Dini et al., 1996). *In situ* analyses showed that intracellular GSH is completely extruded in cells undergoing apoptosis by cleavage, whereas it is fully maintained in apoptotic budding cells (De Nicola et al., 2006). Accordingly, *in situ* measurement of ROS levels showed that only cells in cleavage develop oxidative stress (Celardo et al., 2011). GSH depletion, and the consequent oxidation, is not only concomitant to, but is the determinant of the cleavage route, since inhibition of the sinusoidal GSH transporters completely prevents the appearance of the apoptotic cells displaying the budding morphology (De Nicola et al., 2006).

PRO-SURVIVAL PATHWAYS INDUCED BY GSH DEPLETION

GSH, as a co-factor of the glutathione-S-transferase set of enzymes, plays a major role in the detoxification of cells from exogenous molecules, including pharmaceutical drugs. The depletion of GSH, achieved in clinics with the specific GSH synthesis inhibitor buthionine sulphoxymine (BSO), is an important strategy to improve drug efficacy in anticancer therapies, since many aggressive tumor cells are very efficient in drug extrusion (multi-drug resistant, MDR), thereby nullifying the effects of chemotherapies. In these cases BSO acts a chemosensitizer, since GSH depletion allows bypassing the MDR obstacle, achieving efficacious levels of antitumor drug within MDR cells (Fojo and Bates, 2003).

BSO is a specific inhibitor of glutathione synthase, thereby in BSO-treated cells the GSH pool is consumed but not replaced, and its level declines according to the consumption rate, with a kinetics that depends on the oxidative status of the cells. In any case, the depletion is much slower compared with what occurs during apoptosis (hours vs. minutes). This slow rate gives time to the BSO-treated cells to adapt to the progressively oxidizing environment, setting up stress response pathways aimed at increasing cell survival (Rahman et al., 2005; Ishii and Mann, 2014). Such responses can be very robust, and in non-MDR tumor cells BSO, rather than acting as a chemo-sensitizer, paradoxically potentiates cell survival, thereby decreasing the pro-apoptotic efficiency of anti-tumor drugs (D'Alessio et al., 2004).

The survival responses to GSH depletion include the transcriptional activation of genes encoding for cell protective proteins, *via* the nuclear factor kappaB (NF-kappaB) (Filomeni et al., 2005) or the NF-E2-related factor 2 (Nrf-2) (Lee et al., 2008) transcriptional activators.

NF-kappaB is a transcription complex that in response to many insults, including oxidative stress, mediates the trans-activation of key cell protective proteins (Morgan and Liu, 2011). BSO-mediated activation of NF-kappaB leads to multiple cell-protective responses, such as the trans-activation of heat shock protein 27, which stops the apoptotic signal after Bax translocation and cytochrome c release (Filomeni et al., 2005), and the main anti-apoptotic protein Bcl-2. Bcl-2 is over-expressed in BSO-treated cells via transcriptional activation, both in tumor cells (D'Alessio et al., 2004) and primary lymphocytes (Cristofanon et al., 2006) and monocytes (Cristofanon et al., 2008). The increased Bcl-2 synthesis compensates for, and overcome, the faster catabolism of the Bcl-2 protein occurring in cells with low GSH levels (Meredith et al., 1998; D'Alessio et al., 2004), increasing the intracellular Bcl-2 levels up to three-four folds (D'Alessio et al., 2004). This is achieved through the activation of the so-called non-canonical NF-kappaB pathway by a two-step process. First, as a fast response to GSH depletion, a ternary disulfide complex forms between two subunits of the NF-kappaB component p50 and its tutoring protein Bcl-3 (Cristofanon et al., 2009). Second, the complex is then activated *via* a mechanism dependent on the mitogen-activated protein p38, a kinase that requires high ROS levels (Filomeni et al., 2003); this occurs as a consequence of GSH depletion only several hours later (Limón-Pacheco et al., 2007; Cristofanon et al., 2009).

Nrf-2 is a transcription factor activated by redox imbalance via the removal of its redox-sensitive anchor Keap1 (Kundu and Surh, 2010), and is devoted to the transcription of genes encoding for glutathione metabolism and antioxidant enzymes (Li and Kong, 2009; Ramprasath and Selvam, 2013), thus providing cells with extra antioxidant defense, and reducing the apoptotic outcome. It was found that murine embryonic fibroblasts survive to BSO treatment due to a strong Nrf-2 response, whereas Nrf-2 deficient cells, or cells treated with Nrf2 siRNA became sensitive to BSO-induced apoptosis (Lee et al., 2008).

Therefore, the straightforward use of BSO as coadjuvant in anticancer therapies should be limited to tumors bearing MDR cells. Importantly, non-MDR cells could develop chemo-resistance as a result of BSO treatment, unless they are targeted with specific RNA interfering agents to prevent the over-expression of survival proteins.

CONCLUSIONS

Summarizing, GSH depletion may promote either pro-apoptotic or pro-survival pathways depending on the kinetic of depletion; swift GSH extrusion activates

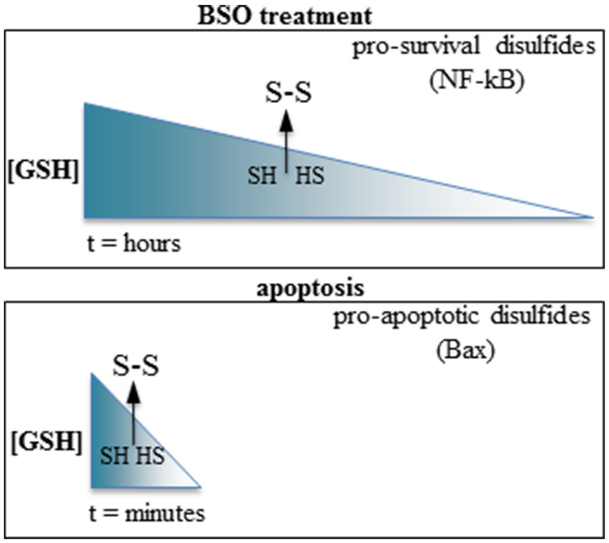

FIGURE 1 | Slow vs. fast GSH depletion produce opposite effects on cell survival. Top: BSO induce slow GSH depletion (hours); the disulfides formed after reaching a threshold level of redox imbalance possess a cell-survival activity (e.g., the p50 subunit of NF-kappaB). **Bottom:** the very rapid GSH extrusion occurring in apoptosis promote the formation of pro-apoptotic disulfides (e.g., Bax). The arrows indicate a hypothetical threshold level of GSH depletion after which sulfhydryls (SH, HS) are oxidized to disulfides (S-S) in each condition.

pro-apoptotic pathways, whereas slower GSH depletion activates pro-survival pathways (**Figure 1**). Intriguingly, both types of response require increased ROS levels and formation of inter-protein disulfides to be activated. The reactivity of cysteines depends on the surrounding aminoacids determining protein conformation, and each inter-protein cystine forms at fixed levels of oxidation of the cell microenvironment. It is conceivable that the reactive cysteines of the proteins involved in the opposite responses (e.g., the cell-protective NF-kappaB component p50 vs. the pro-apoptotic Bax) may be differently modulated according to the swiftness of the redox imbalance, possibly differently relying in the tutoring effect of thiol-modifying enzymes such as thioredoxin or protein disulfide isomerase.

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Glutathione synthesis is compromised in erythrocytes from individuals with HIV

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We demonstrated that the levels of enzymes responsible for the synthesis of glutathione (GSH) such as glutathione synthase (GSS), glutamate-cysteine ligase-catalytic subunit (GCLC), and glutathione reductase (GSR) were significantly reduced in the red blood cells (RBCs) isolated from individuals with human immunodeficiency virus (HIV) infection and this reduction correlated with decreased levels of intracellular GSH. GSH content in RBCs can be used as a marker for increased overall oxidative stress and immune dysfunctions caused by HIV infection. Our data supports our hypothesis that compromised levels of GSH in HIV infected individuals' is due to decreased levels of GSH-synthetic enzymes. The role of GSH in combating oxidative stress and improving the functions of immune cells in HIV patients' indicates the benefit of an antioxidant supplement which can reduce the cellular damage and promote the functions of immune cells.

Keywords: glutathione, HIV, GSS, GCL, GSR

INTRODUCTION

Roughly 34 million people around the world are infected with human immunodeficiency virus (HIV). Since its first reporting in 1981, the beginning of an epidemic, 60 million people have contracted HIV and an estimated 30 million have died due to HIV related causes (World Health Organization, 2010). HIV infection is associated with a wide range of different opportunistic infections that are usually the prime suspect for patients' poor survival. Among the array of opportunistic infections, one of the leading life-threatening infection common among HIV positive individuals with compromised immune system is *Mycobacterium tuberculosis*. Especially in developing countries, as many as eighty percent of people with AIDS are at risk of developing tuberculosis (TB) (World Health Organization, 2009). HIV's primary targets *in vivo* are blood monocytes, CD4 T lymphocytes, and resident macrophages. Due to HIV's high affinity for infecting and killing CD4+ T lymphocytes, cell-mediated immunity is drastically lowered. This results in greater probability for opportunistic infections, primarily *M. tuberculosis* (Levy, 1993; Pantaleo et al., 1993; Droge and Holm, 1997; Herzenberg et al., 1997).

Glutathione (GSH) is a major component involved in the control and maintenance of cellular redox state and cellular homeostasis (Griffith, 1999). In addition, GSH is also important in an array of cellular functions such as protein synthesis, transport across membranes, receptor action, and cell growth (Griffith, 1999). As a natural antioxidant, GSH scavenges peroxide species. Low levels of GSH have been shown to play a role in the apoptosis of CD4+ T cells, which is the major pathology of the HIV infection, therefore signifying the importance of GSH (Levy, 1993; Pantaleo et al., 1993; Droge and Holm, 1997; Herzenberg et al., 1997).

Glutathione is produced by almost all cell types and are present in two forms, reduced (*r*GSH) and oxidized (GSSG). *r*GSH is synthesized by two different mechanisms. *De novo* synthesis of *r*GSH occurs in a two-step process mediated by two different enzymes, glutamate-cysteine ligase (GCL) and glutathione synthase (GSS). *r*GSH is also synthesized via the reduction of GSSG by glutathione reductase (GSR; Staal, 1998). In this study, we went beyond the innate immune response components and investigated the changes in the levels of GSH in red blood cells (RBCs) isolated from individuals with HIV infection. We hypothesized that compromised levels of GSH in HIV-infected individuals is due to decreased levels of enzymes that are involved in the synthesis of GSH. Since RBCs are systemically present in abundance, we tested our hypothesis by determining the extent to which the levels of GSH-synthetic enzymes are compromised in RBCs derived from individuals with HIV infection and correlating decreased levels of GSH-synthetic enzymes with deficiency in the levels of GSH.

MATERIALS AND METHODS

SUBJECTS

The protocol was approved by Institutional Review Board with the requirement that each volunteer recruited would need to be given a consent form that described the basis and the procedures of the study. A signed informed consent from each volunteer that agreed to participate was obtained. A total of 16 volunteers (eight healthy subjects and eight individuals with HIV infection) were recruited for the study. Individuals with HIV infection were recruited from the Foothills AIDS project. Healthy subjects without HIV infection

or a history of TB were recruited from the staff of Western University of Health Sciences. All HIV-infected volunteers had been diagnosed with HIV-1, were taking some form of anti-retroviral treatment, and had CD4+ T-cell counts between 271 and 1415 cells per mm^3. Thirty five milliliters (mL) of blood was drawn once from both healthy volunteers and individuals with HIV infection.

ERYTHROCYTE ISOLATION

Red blood cells were isolated from whole blood by density gradient centrifugation with FICOLL-Paque (GE Healthcare, 17-440-02). RBCs that aggregated as the bottom layer were collected and stored at −20°C in a cell lysis/protein storage buffer [20 mM Tris, 100 mM NaCl (Amresco, N653), 1X protease inhibitor cocktail (Amresco, M221)] for western blot analysis.

GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS IN RBC LYSATES FROM HEALTHY AND HIV$^+$ SUBJECTS

Total protein content was determined using Coomassie blue colorimetric assay (Thermo Scientific, PI-23200). 200 μg of total RBC proteins per sample were separated via denaturing polyacrylamide electrophoresis (12%). Separated proteins were transferred to a Polyvinylidene fluoride membrane (GE, PV4HY00010) by electroblotting. Membranes were blocked for 1 h at room temperature in tris buffered saline with tween 20 (TBST) and 5% non-fat dry milk followed by three washes (15 min for each wash) in TBST with mild shaking. The membranes were then incubated with a primary antibody overnight at 4°C in TBST with gentle shaking. The primary antibodies used were mouse anti-human GSS (1:1000, Abcam, ab5513), mouse anti-human GSR (1:500, Abcam, ab55075), and rabbit anti-human glutamate-cysteine ligase-catalytic subunit (GCLC; 1:250, Abcam, ab40929). Following overnight incubation with the primary antibodies, membranes were washed five times for 15 min in TBST with mild shaking. Washed membranes were incubated with a secondary antibody conjugated with horse radish peroxidase, anti-mouse (1:1000, Abcam, ab7064), or anti-rabbit (1:1000, Abcam, ab72465) in TBST for an hour at room temperature. Membranes were washed again five times for 15 min in TBST with mild shaking. Chemiluminescent substrate was applied to the membranes which were then exposed to an x-ray film (Genemate, F-9024) and developed in a dark room. Digital images of the immunoblots were captured using a Versadoc gel imaging system (Bio-rad, 4000 MP). Densitometric analysis of the images was performed using ImageJ, a free software program available from the National Institutes of Health (http://rsbweb.nih.gov/ij/).

ASSAY OF GSH LEVELS IN RBCs FROM HEALTHY AND HIV$^+$ SUBJECTS

Glutathione concentrations were measured in RBCs isolated from healthy and HIV$^+$ individuals by spectrophotometry using a colorimetric assay kit (Arbor Assays, K006-H1). RBCs were suspended in an ice cold 5% 5-sulfosalicylic acid dihydrate solution (MP Biomedicals, 160001-4924H). Supernatants collected after centrifugation were analyzed for the total GSH as per the manufacturer's instructions. All GSH measurements were normalized with total protein concentrations.

STATISTICAL ANALYSIS

Statistical analysis of the data was carried out using GraphPad Prism 6. The data was analyzed by comparing the means of $n = 8$ individuals (unless otherwise specified) using unpaired student's t-tests. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Glutathione is a tripeptide made of glutamine, cysteine, and glycine. In the *de novo* synthesis of GSH, glutamine is linked to cysteine by GCL to form -glutamylcysteine (Griffith, 1999). Then GSS links the dipeptide -glutamylcysteine to glycine to form the final GSH molecule (Griffith, 1999). The GSH redox system plays a major role in ridding the body of oxidative stress and restoring homeostasis (Griffith, 1999). To elicit antioxidant effects, GSH is converted to oxidized glutathione (GSSG) by glutathione peroxidase (GPx). GSSG can be converted back to GSH by GSR (Staal, 1998). It is important to note that only free GSH has antioxidant effects. On the other hand, GSSG lacks antioxidant functions and is a byproduct of the scavenging activity of GSH (Staal, 1998; Griffith, 1999). GSH/GSSG ratio should be maintained to optimize the GSH redox system. GCL, the rate-limiting enzyme of GSH synthesis, is composed of a catalytic subunit (GCLC) and a modulating subunit (GCLM). GCLC is the component that performs the amino acid linkage between glutamine and cysteine, whereas GCLM modulates the activity of GCLC (Huang et al., 1993).

It has previously been reported that GSH levels in the plasma, erythrocytes, and peripheral blood mononuclear cells (PBMC) of HIV$^+$ individuals are compromised (Sbrana et al., 2004; Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). The goal of our study is to characterize the causes for diminished levels of GSH in HIV infected individuals by determining the extent to which the levels of GCLC, GSS, and GSR are decreased in RBCs isolated from individuals with HIV infection compared to healthy subjects. Measurement of GSS and GCLC revealed a significant decrease in the levels of these enzymes present in RBCs of HIV-infected individuals compared to healthy subjects (**Figures 1** and **2**). Both GSS and GCLC are crucial enzymes that are involved in the catalytic rate limiting step and second step reaction, respectively, in the biosynthesis of GSH (Staal, 1998; Griffith, 1999; Morris et al., 2012, 2013). We also observed a significant decrease in the expression of GSR in RBCs isolated from HIV positive subjects (**Figure 3**). This explains the reason for decreased levels of GSH and the consequences related to the GSH deficiency such as loss of immune function observed in HIV patients (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Reduced expressions of GSH synthesis enzymes in RBCs from individuals with HIV infection was accompanied by decreased levels of total GSH (**Figure 4**).

HIV$^+$ individuals were also found to have increased levels of TGF-β in their plasma and macrophage supernatants (Morris et al., 2012). Moreover, TGF-β is known to block the production of GCLC which leads to decreased GSH synthesis (Morris et al., 2012). HIV-1 transactivator protein (TAT) decreases the amount of GSH present in mice through the modulation of GSH biosynthetic enzymes (Choi et al., 2000).

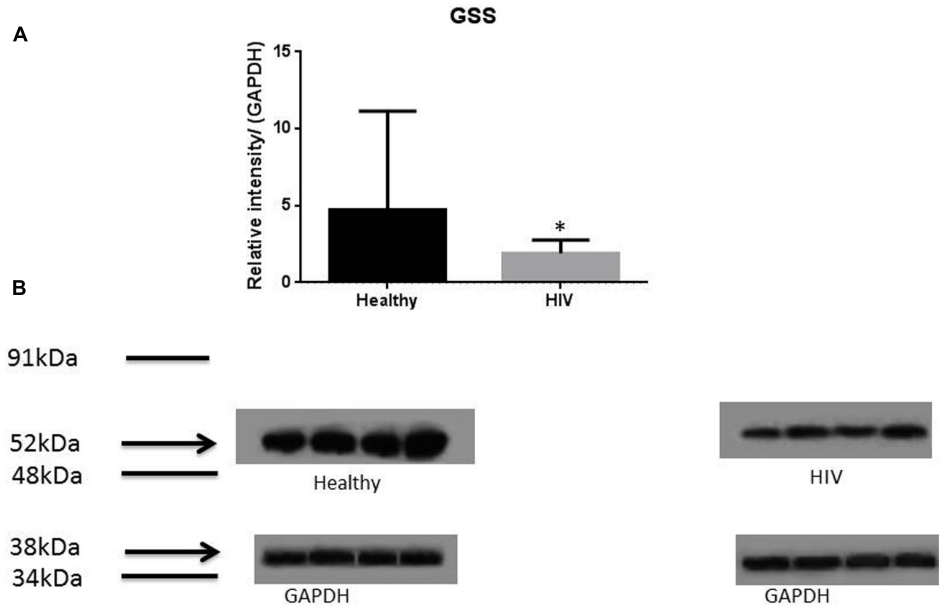

FIGURE 1 | Quantifying of GSS enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of GSS, the enzyme that is involved in the second step of GSH synthesis. Electrophoresis and Western blot were used. There was a significant decrease of GSS in the RBC of HIV-infected individuals compared to healthy individuals. Data in **(A)** represents significant difference in the levels of GSS between RBCs isolated from eight healthy individuals and eight individuals with HIV infection ($*p \leq 0.05$). **(B)**: Illustrates representative images of GSS and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

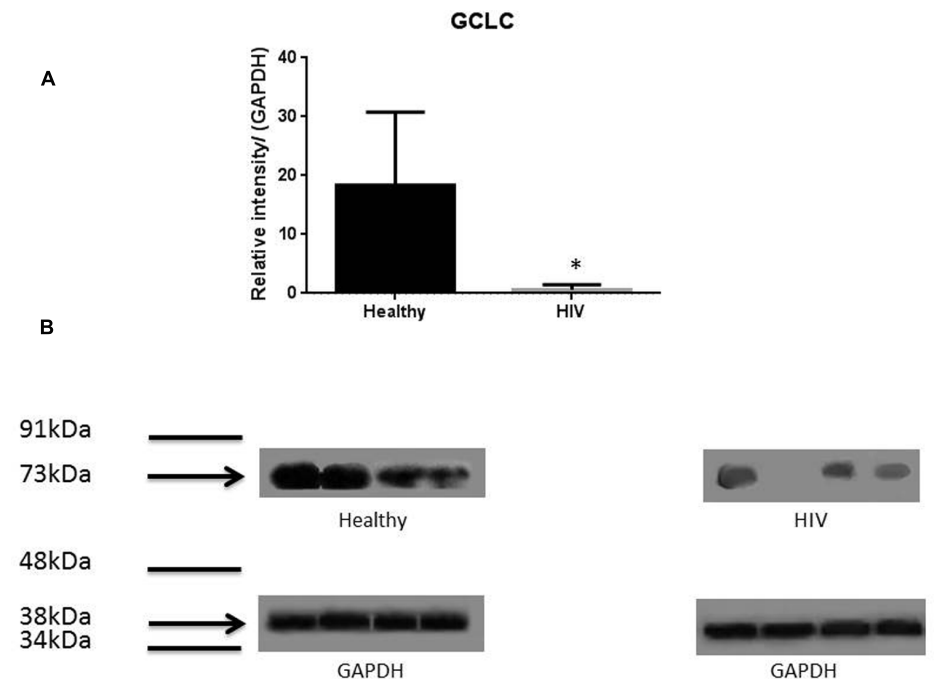

FIGURE 2 | Quantifying of GCLC enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of the catalytic subunit of GCLC that is responsible for the linkage between glutamine and cysteine in the first step of GSH synthesis. Electrophoresis and Western blot were used. GCLC levels were significantly lower in the RBC of HIV-infected individuals compared to healthy subjects. Data in **(A)** represents significant difference in the levels of GCLC between RBCs isolated from eight healthy individuals and eight individuals with HIV infection ($*p \leq 0.05$). **(B)**: Illustrates representative images of GCLC and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

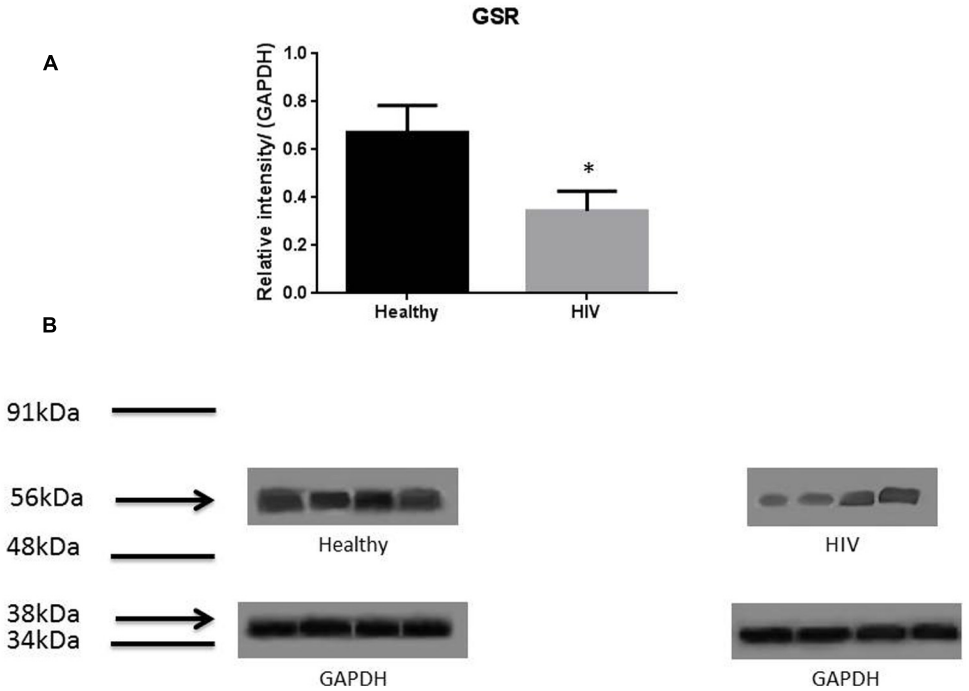

FIGURE 3 | Quantifying of GSR enzyme levels in Healthy and HIV-infected subjects. Red blood cell samples were separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of GSR. GSR converts oxidized glutathione (GSSG) to glutathione (GSH). The GSR levels in the RBC of HIV-infected individuals are lower than healthy individuals. Electrophoresis and Western blot were used. GSR levels in HIV-infected individuals are lower compared to healthy. Data in **(A)** represents significant difference in the levels of GSR between RBC isolated from five healthy individuals and five individuals with HIV infection ($*p \leq 0.05$). **(B)**: Illustrates representative images of GSR and their corresponding GAPDH bands from four healthy subjects and four individuals with HIV infection.

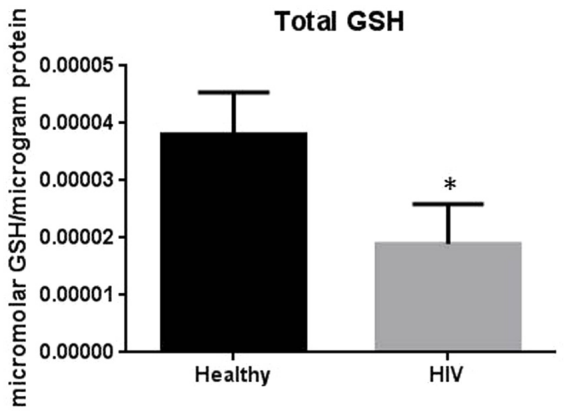

FIGURE 4 | Assay of GSH levels in RBCs from healthy subjects and individuals with HIV infection. Analysis of total GSH which includes both free GSH and GSSG indicated significantly higher levels of GSH in healthy individuals compared to HIV positive subjects. Data in **Figure 4** represents significant difference in the levels of total GSH between RBCs isolated from five healthy individuals and five individuals with HIV infection ($*p$=0.05).

TAT also increases free radical production. Therefore, marked increase in oxidative stress along with increased levels of TGF-β lead to the compromised levels of GSH synthesis enzymes. The master transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) regulates the expression of antioxidant and phase II-metabolizing enzymes by activating the antioxidant response element (ARE) and thereby protects cells and tissues from oxidative stress. The Nrf2 gene binding to the ARE results in the upregulation of GSH synthesis enzymes such as GCLC, GCLM, and GSR. New findings argue that HIV-1-related proteins downregulate Nrf2 expression and/or activity within the alveolar epithelium, which in turn impairs antioxidant defenses and barrier function, thereby rendering the lung susceptible to oxidative stress and injury (Fan et al., 2013). Furthermore, this study suggests that activating the Nrf2/ARE pathway with the dietary supplement sulforaphane could augment antioxidant defenses and lung health inHIV-1-infected individuals (Fan et al., 2013).

We have previously reported that the virulent laboratory strain of *M. tuberculosis* H37Rv is sensitive to GSH at physiological concentrations (5 mM) when grown *in vitro* (Venketaraman et al., 2005). Thus, GSH has direct antimycobacterial activity, functioning as an effector molecule in innate defense against *M. tuberculosis* infection (Venketaraman et al., 2005; Dayaram et al., 2006). We recently reported that GSH is integral in facilitating the control of intracellular growth of *M. tuberculosis* in human macrophages (Venketaraman et al., 2005; Dayaram et al., 2006; Morris et al., 2012, 2013). These results further confirm that GSH has direct antimycobacterial activity and unfolds a novel and potentially important innate defense mechanism adopted by

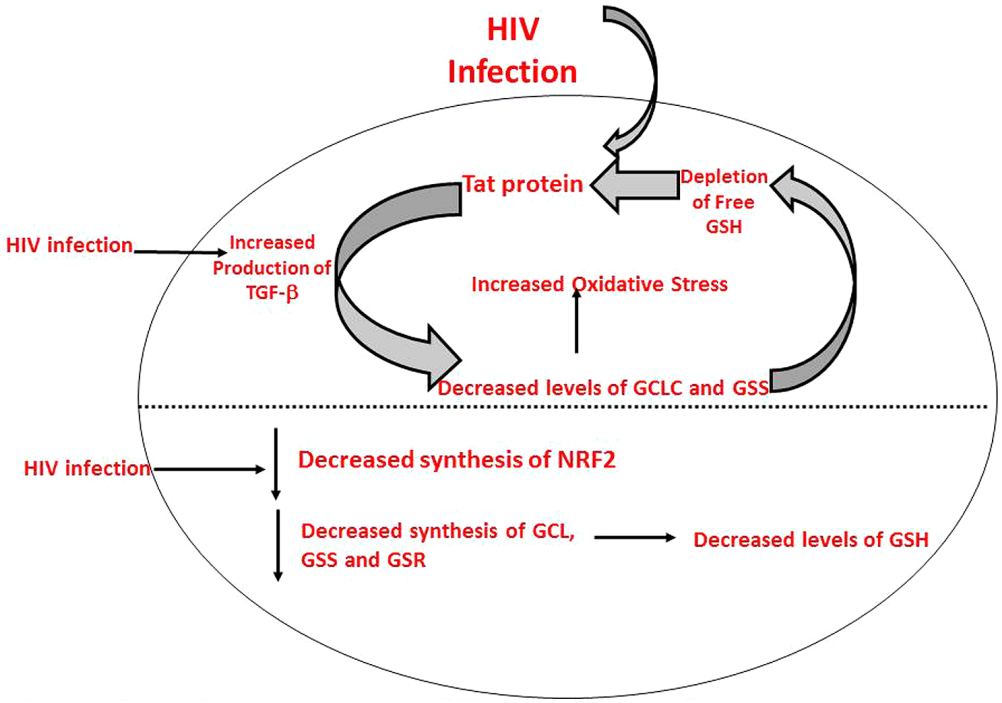

FIGURE 5 | Model illustrating causes for decreased levels of GSH in individuals with HIV infection.

human macrophages to control *M. tuberculosis* infection. We also demonstrated that GSH in combination with cytokines such as IL-2 and IL-12 enhances the functional activity of natural killer (NK) cells to inhibit the growth of *M. tuberculosis* inside human monocytes (Millman et al., 2008; Guerra et al., 2012). Importantly, data from our most recent studies indicate that GSH activates the functions of T lymphocytes to control *M. tuberculosis* infection inside human monocytes (Guerra et al., 2011). These results indicate that GSH inhibits the growth of *M. tuberculosis* by both direct antimycobacterial effects as well as by activating the functions of immune cells (Venketaraman et al., 2005; Dayaram et al., 2006; Millman et al., 2008; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). We also reported that the GSH concentrations were significantly lower in macrophages, NK, and T cells isolated from individuals with HIV infection compared to healthy subjects (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Decreased levels of GSH in macrophages, NK ,and T cells derived from individuals with HIV infection was accompanied by diminished control of intracellular *M. tuberculosis* infection (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013). Our group is a pioneer in reporting that GSH levels were decreased in macrophages, T cells, and NK cells from individuals with HIV infection and correlating decreased GSH levels with impaired innate and adaptive immune responses against *M. tuberculosis* infection (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013).

In this study we investigated the cause for decreased levels of GSH in individuals with HIV infection by quantifying the levels of GSS, GCLC, and GSR in the RBCs derived from healthy subjects and individuals with HIV infection. The results of the Western Blot indicate that there is a significant difference in the levels of GSS, GCLC, and GSR between HIV-infected individuals and healthy individuals, which supports our hypothesis that individuals with HIV infection have lower concentrations of enzymes that are responsible for both *de novo* synthesis of GSH and conversion of GSSG to GSH (**Figures 1–3**). In addition, our results indicate that there is a significant decrease in the levels of total GSH in the RBCs derived from HIV-infected individuals (**Figure 4**). Overall, these significant findings indicating lower levels of GSS, GCLC, and GSR in HIV-infected individuals support our hypothesis and contribute to previous findings that there are lower levels of GSH in HIV-infected individuals than healthy individuals (**Figure 5**). Observations from the current study combined with our previous findings strongly suggest that liposomal formulations of GSH can be used as a possible supplement to current HIV treatments since they can provide complete *r*GSH molecules, bypassing the cellular machinery for GSH production. Liposomal formulations containing GSH can be more effective in supplementing the intracellular *r*GSH and restoring the immune cell functions including the antimycobacterial activity in macrophages from HIV patients at concentrations lower than NAC (Venketaraman et al., 2006; Guerra et al., 2011, 2012; Morris et al., 2012, 2013).

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Inhibiting lung lining fluid glutathione metabolism with GGsTop as a novel treatment for asthma

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Asthma is characterized by airway inflammation. Inflammation is associated with oxidant stress. Airway epithelial cells are shielded from this stress by a thin layer of lung lining fluid (LLF) which contains an abundance of the antioxidant glutathione. LLF glutathione metabolism is regulated by γ-glutamyl transferase (GGT). Loss of LLF GGT activity in the mutant GGT^{enu1} mouse causes an increase in baseline LLF glutathione content which is magnified in an IL-13 model of allergic airway inflammation and protective against asthma. Normal mice are susceptible to asthma in this model but can be protected with acivicin, a GGT inhibitor. GGT is a target to treat asthma but acivicin toxicity limits clinical use. GGsTop is a novel GGT inhibitor. GGsTop inhibits LLF GGT activity only when delivered through the airway. In the IL-13 model, mice treated with IL-13 and GGsTop exhibit a lung inflammatory response similar to that of mice treated with IL-13 alone. But mice treated with IL-13 and GGsTop show attenuation of methacholine-stimulated airway hyper-reactivity, inhibition of Muc5ac and Muc5b gene induction, decreased airway epithelial cell mucous accumulation and a fourfold increase in LLF glutathione content compared to mice treated with IL-13 alone. Mice treated with GGsTop alone are no different from that of mice treated with saline alone, and show no signs of toxicity. GGsTop could represent a valuable pharmacological tool to inhibit LLF GGT activity in pulmonary disease models. The associated increase in LLF glutathione can protect lung airway epithelial cells against oxidant injury associated with inflammation in asthma.

Keywords: GGsTop, glutathione, lung lining fluid, asthma, IL-13

INTRODUCTION

The lung epithelial surface is bathed by a thin, continuous layer of fluid referred to as lung lining fluid (LLF). Its function has been described as a shield at the air–liquid interface to protect the lung epithelium from environmental stress (Cantin et al., 1987). The LLF provides an aqueous medium for the exchange of molecules within the surfactant system, a supportive milieu for the alveolar macrophage, a protective surface for the alveolar septum, and thin component of the blood-diffusion distance (Bastacky et al., 1995). Among the small molecule antioxidants present in LLF, glutathione (GSH) has received particular attention because its concentration exceeds that of the blood by 100-fold and its abundance exceeds that of LLF glutathione disulfide (GSSG; Cantin et al., 1987). In addition, the lung overall utilizes more glutathione than any other organ (Martensson et al., 1989). Cellular glutathione synthesis and extracellular export and metabolism impact overall glutathione homeostasis (Lieberman et al., 1996; Dalton et al., 2000; Shi et al., 2000). Metabolism of glutathione is regulated by gamma-glutamyl transferase (EC 2.3.2.2, GGT) and provides a cysteine source for cellular glutathione resynthesis (Hanigan and Ricketts, 1993). GGT is present in the lung as a soluble enzyme in association with surfactant phospholipids and controls turnover of the extracellular pool of glutathione in LLF (Joyce-Brady et al., 1994; Jean et al., 2002). The biological role of the LLF glutathione pool and its metabolism by GGT was revealed through our studies in a genetic mouse model of GGT deficiency, the GGT^{enu1} mutant mouse (Harding et al., 1997; Jean et al., 1999, 2002; Lowry et al., 2008). In a IL-13-driven model of experimental allergic asthma, GGT^{enu1} mutant mice were protected against asthma, despite the presence of cellular glutathione deficiency and oxidant stress in lung airway epithelial cells (Jean et al., 2002; Lowry et al., 2008). IL-13 is a well described pro-inflammatory cytokine that drives inflammation in a mouse model of experimental asthma (Grunig et al., 1998; Zhu et al., 1999). This cytokine-driven model evokes a similar degree of inflammatory cell infiltration into the lungs of normal mice and GGT^{enu1} mutant mice, thereby allowing study of their lung response to inflammation (Lowry et al., 2008; Joyce-Brady et al., 2012). We found that the GGT deficient GGT^{enu1} mouse was protected against asthma in this model by an increased size of the extracellular LLF glutathione pool which buffered oxidant species derived from inflammatory cells, and preserved airway epithelial cell barrier function. In turn, induction of mucin gene expression, mucous production and airway hyperreactivity were

dramatically decreased in GGT^{enu1} mutant mice. Control mice did develop asthma but could be protected against this by inactivating their LLF GGT activity pharmacologically with acivicin, an irreversible GGT enzyme inhibitor. These data revived interest in the extracellular LLF glutathione pool and suggested that GGT activity in LLF is a novel target to treat asthma (Lowry et al., 2008; Joyce-Brady et al., 2009; Joyce-Brady and Hiratake, 2011).

Human pharmacotherapy with acivicin, however, is limited by neurotoxicity and cytotoxicity caused by an extended inhibitory activity against a number of other glutamine-dependent biosynthetic enzymes (Hidalgo et al., 1998; Joyce-Brady and Hiratake, 2011). These limitations led to the rational design of novel class of glutamate analogous γ-phosphono diesters as mechanism-based inhibitors of GGT. These compounds are based on stable analogs of the enzyme transition state and inhibit GGT activity with greater potency and specificity than acivicin (Han et al., 2006, 2007; Joyce-Brady and Hiratake, 2011). We focused on the lead compound from this class, now commercially available as GGsTop, for three reasons: lack of toxicity *in vivo*, physical stability *in vitro* and extended inhibitory activity against mammalian as well as bacterial GGT (Han et al., 2006, 2007). We hypothesized that GGsTop could attenuate asthma in the IL-13-driven model of allergic airway inflammation in mice and represent a valuable alternative to acivicin as a clinical therapeutic.

MATERIALS AND METHODS

INHIBITORY ACTIVITY OF GGsTop *IN VIVO*

A crystalline form of GGsTop was synthesized according to the previous report (Han et al., 2007). GGsTop is freely soluble in distilled water so a 10 mM stock solution was prepared, stored at −20°C and aliquoted for experiments. The compound is now available commercially as GGsTop^{TM} from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). In preliminary studies, GGsTop was assessed for its ability to inhibit GGT enzyme activity *in vivo* in mouse lung and serum at 0.5 mg/kg and 5 mg/kg. GGsTop was delivered via the peritoneum or through the trachea in a volume of 100 μL. GGT activity was assessed 2 h and 24 h afterward after delivery in bronchoalveolar lavage fluid and serum. Controls received phosphate buffered saline (PBS). GGT enzyme activity was assessed at room temperature using a standard method with γ-glutamyl-*p*-nitroanilide as substrate (Hughey and Curthoys, 1976; Joyce-Brady et al., 1994). Data are expressed as a percentage of the control group.

MOUSE MODEL OF IL-13-DRIVEN EXPERIMENTAL ALLERGIC ASTHMA

C57BL/6 mice were housed in the Laboratory and Animal Science Center at Boston University School of Medicine under guidelines approved by the local Institutional Animal Care and Utilization Committee in protocol number AN-14284. Animals were fed Purina mouse chow and allowed access to water *ad libitum*. In each experiment, two groups of 6–8 mice were pre-dosed with saline or GGsTop dissolved in PBS via the trachea on day 0. Thereafter 3–4 mice were treated every 24 h for three successive days with either saline alone (control), saline with IL-13, saline with GGsTop, or saline with IL-13 and GGsTop. The IL-13 dose was five micrograms and the GGsTop was 0.5 mg/kg. On the final day, airway resistance (Rn) was measured using an intratracheal catheter on a Scireq flexivent apparatus (SCIREQ, Montreal, PQ, Canada) under appropriate anesthesia with pentobarbital. The tidal volume was 6–7 ml/kg, the positive-end expiratory pressure was 3 cm H_2O, and the respiratory rate was 150 breaths/min. Basline airway resistance was measured after delivery of nebulized saline followed by increasing doses of nebulized methacholine Mch (5, 15, and 25 mg/ml). Data were normalized to the baseline level. Thereafter, lungs were either frozen to isolate RNA, inflation-fixed with paraformaldehyde for histology or lavaged to collect bronchoalveolar lavage fluid (Lowry et al., 2008).

RNA ANALYSIS

Total RNA was isolated from frozen lung tissue, quantified by spectrophotometry and assayed for messenger RNA transcript abundance using standard real time PCR methodology as described (Lowry et al., 2008). The messenger RNA included two mucin genes, Muc5ac and Muc5b (Lowry et al., 2008) and the cytokine IL33 (Moffatt et al., 2010).

HISTOLOGY AND CYTOLOGY

Standard techniques were used to fix lung tissue in 4% paraformaldehyde and to embed in paraffin. Sections were stained with periodic acid-Schiff (PAS) stain as described (Lowry et al., 2008). Cells in bronchoalveolar lavage were collected with low speed centrifugation, transferred to glass slides, stained with Giemsa, and visualized under light microscopy.

BRONCHOALVEOLAR LAVAGE

Mouse lungs were inflated with 500 μL of PBS and centrifuged to sediment cells. The cell pellet was used to prepared cytospin preps and stained with Geimsa. The cell-free supernatant was combined with a sulfhydryl preserving solution and assayed for glutathione content by HPLC and normalized based on the urea dilution method as described previously (Jean et al., 2002; Lowry et al., 2008). Total glutathione content is reported.

STATISTICS

Nominal data are presented as means with SE and analyzed by ANOVA and Dunnet's or Bennett's *post hoc* test as indicated. P values <0.05 were considered significant.

RESULTS

LUNG LINING FLUID GGT ACTIVITY IS INHIBITED BY GGsTop ONLY WITH INHALATION

Lung lining fluid GGT activity was not inhibited with GGsTop delivery thru the peritoneum at the 0.5 mg/kg dose (**Figure 1A**, $n = 2$) nor at the 5 mg/kg dose (same pattern, data not shown). This route did inhibit GGT activity in the blood (**Figure 1B**). The inhibition at 2 h was dose dependent with serum activity decreasing to 60% of control with 0.5 mg/kg ($p < 0.05$, $n = 3$) and 90% of control with 5 mg/kg ($p < 0.05$, $n = 3$). By 24 h there was a regain of serum GGT activity to 100% of control with the 0.5 km/kg dose ($n = 2$) and 67% of control with the 5 mg/kg dose ($n = 2$).

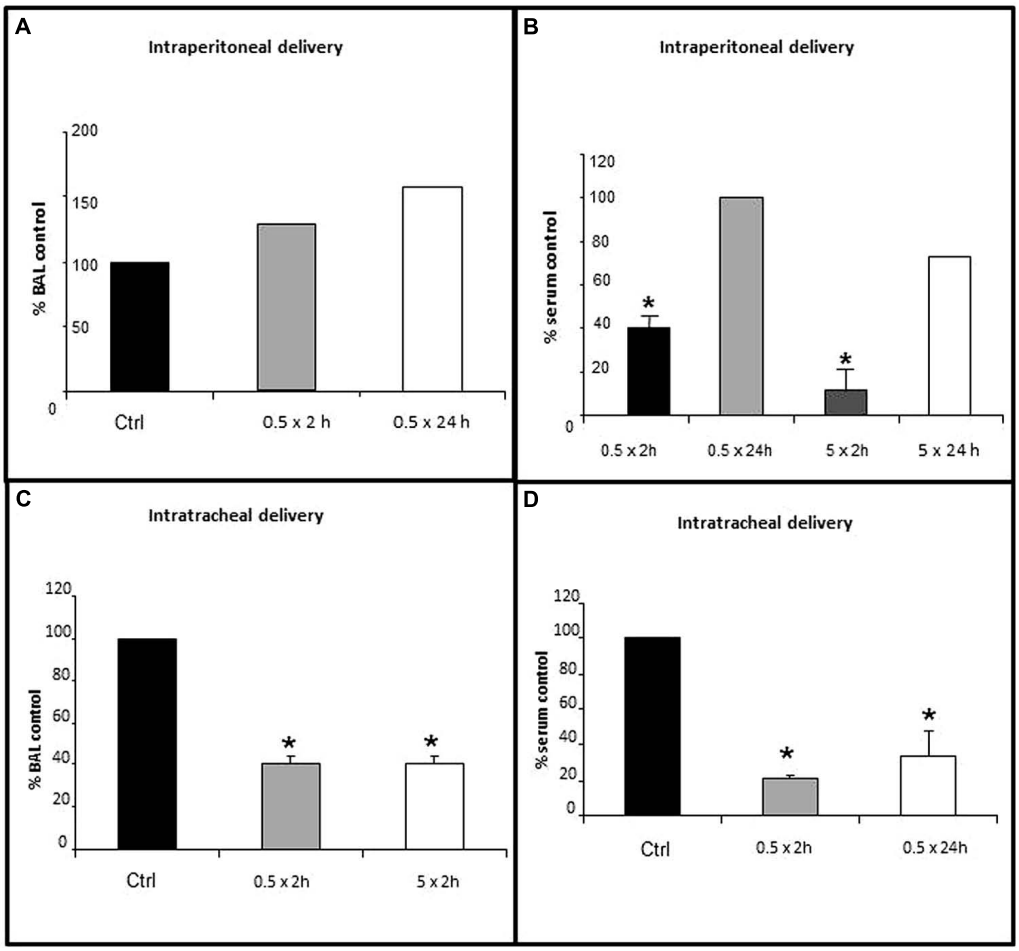

FIGURE 1 | Inhibition of lung lining fluid (LLF) GGT activity with delivery of GGsTop via the trachea, but not the peritoneum. GGsTop was delivered thru the trachea and the peritoneum at doses of 0.5 mg/kg and 5 mg/kg. GGT activity was assessed in broncho-alveolar lavage fluid **(A,C)** and serum **(B,D)** as described in "Materials and Methods." Data are expressed as percentage versus the control group. Significant differences at $p < 0.05$ are denoted by asterisks ($n = 3$). The data in 1A and those in 1B at 24 h are averages from $n = 2$ replicates.

Two hours after GGsTop was delivered via the trachea, the 0.5 mg/kg dose and the 5 mg/kg dose each inhibited GGT enzyme activity in LLF to 40% of control ($p < 0.05$, $n = 3$, **Figure 1C**). Intratracheal delivery of GGsTop (0.5 mg/kg) also inhibited serum GGT activity to 20% of control at 2 h ($p < 0.05$, $n = 3$) and this decrease was still evident 24 h later (30% of control, $p < 0.05$, $n = 3$, **Figure 1D**). The 0.5 mg/kg dose was used for the IL-13 experiments and delivered through the trachea.

GGsTop ATTENUATES IL-13 INDUCED AIRWAY HYPER-REACTIVITY

Treatment of mouse lung with IL-13 caused a significant, and dose dependent increase in relative airway resistance following methacholine challenge compared to saline treated control mice (**Figure 2**). This pattern was attenuated at each methacholine dose when GGsTop was added to IL-13. The effect was significant at the highest dose ($p < 0.0001$, $n = 9$). Administration of GGsTop alone did not alter the airway resistance parameter versus the control group.

GGsTop AUGMENTS TOTAL GLUTATHIONE IN LUNG LINING FLUID IN THE PRESENCE OF IL-13

Total gutathione was measured in LLF at the end of the IL-13 experiment. In the absence of IL-13, GGsTop did not change LLF glutathione from that of the saline control (**Figure 3A**). IL-13 treatment itself did not change LLF glutathione either. However, treatment with IL-13 and GGsTop increased LLF glutathione by almost fourfold ($p < 0.05$, $n = 3$).

IL-13 DRIVEN INFLAMMATION IS UNAFFECTED BY GGsTop, BUT INDUCTION OF MUCOUS EXPRESSION IS ATTENUATED

Compared to control lung, exposure to IL-13 induced the level of lung mRNA transcript expression for IL-33 by sixfold (**Figure 3B**) and recruited eosinophils to the lung in the absence (**Figure 3C**) and presence (**Figure 3D**) of GGsTop.

IL-13 treatment induced the lung level of messenger RNA transcript expression for Muc5ac 28-fold ($p < 0.05$, $n = 3$, **Figure 4A**) and Muc5b sixfold ($p < 0.05$, $n = 3$, **Figure 4B**) versus control mice treated with saline alone. Compared to PBS treated lung

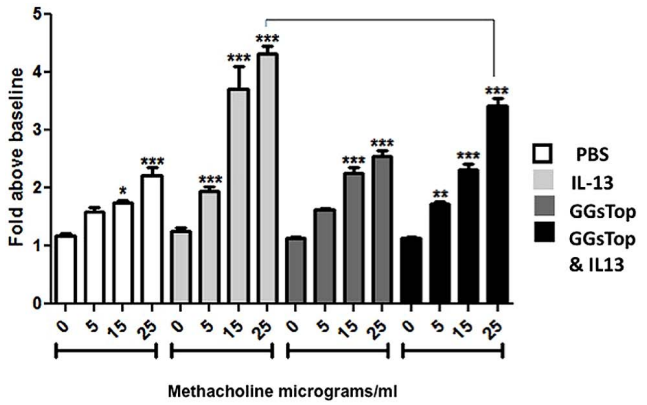

FIGURE 2 | GGsTop attenuates airway hyper-reactivity induced by IL-13. Induction of airway hyperreactivity was measured by assaying airway resistance during graded methacholine challenge as described in "Materials and Methods." Asterisks denote significant increase in airway resistance over baseline in each group ($p < 0.05$). IL-13 treatment increased airway resistance at each methacholine dose compared to saline control ($n = 9$/methacholine dose). Administration of GGsTop alone did not alter the airway resistance parameter versus the control group. Addition of GGsTop to IL-13, however, attenuated airway resistance at each dose of methacholine with a significant decrease at the highest dose ($n = 9$/methacholine dose, $p < 0.0005$).

(**Figure 4C**), PAS stain of IL-13 treated lung showed a diffuse signal for mucous in airway epithelial cells (**Figure 4E**; Lowry et al., 2008). When GGsTop was added to IL-13, induction of messenger RNA transcript for Muc5ac was decreased sevenfold (**Figure 4A**) and that of Muc5b twofold (**Figure 4B**) and PAS stained lung showed only rare airway epithelial cells with a mucous signal (**Figure 4F**). GGsTop treatment alone affected neither mucin gene expression nor mucous production (**Figures 4A,B,D**).

DISCUSSION

Oxidant stress is believed to be important in asthma pathogenesis (Rahman et al., 2006). An inverse relationship has been noted previously in humans between the level of the antioxidant glutathione in LLF and airway hyperreactivity. That is a higher level of lung lining fluid glutathione is associated with lesser degree of airway hyperreactivity (Smith et al., 1993). The antioxidant glutathione is found in abundance in the lining fluid and protects the surface of the lung against oxidant stress (Cantin et al., 1987). LLF glutathione is metabolized by the enzyme GGT, which we showed is also present in lung lining fluid (Joyce-Brady et al., 1994). GGT expression in inducible LLF with inhalation of oxidant gas (Takahashi et al., 1997) and in many different cells in the lung during injury, including neutrophils, (Corti et al., 2012), bronchiolar Clara cells (Takahashi et al., 1997), and type 2 epithelial cells (van Klaveren et al., 1997). GGT-mediated glutathione metabolism provides cells with cysteine at the expense of the lung lining fluid glutathione pool. Interestingly, genetic deficiency of GGT in GGT^{enu1} mice induced an asthma-resistant phenotype in an IL-13-driven model of allergic airway hyperreactivity. Lung lining fluid glutathione content was augmented nearly 10-fold over that of normal mice at baseline, when GGT deficient GGT^{enu1} mice were exposed to this model of cytokine-driven inflammation. This enhanced antioxidant availability buffered inflammation-associated oxidant stress and attenuated airway epithelial cell EGF-receptor activation, induction of mucous hypersecretion and airway hyper-reactivity in GGT^{enu1} mice. Protection against asthma was conferred on normal mice by pharmacologic inhibition of LLF GGT with acivicin. Our data identified GGT as a target to treat asthma. We proposed this as a novel asthma treatment strategy to complement current asthma therapeutics which target inflammation (Lowry et al., 2008).

Our studies in the GGT^{enu1} mouse also demonstrated the value of this animal model in defining a biologic role for extracellular pools of glutathione in combating oxidant stress derived from inflammation. These observations can be translated into normal mice by augmenting their LLF glutathione thru inhibition of GGT enzyme activity (Joyce-Brady and Hiratake, 2011). In our original paper we successfully used acivicin to inhibit GGT activity. This inhibition is irreversible but certain adverse effects are already known to limit clinical acivicin use. It is non-specific and inhibits other glutamine amidotransferases as well as GGT (Joyce-Brady and Hiratake, 2011). It is also neurotoxic *in vivo* as shown in previous human clinical trials as a cancer therapeutic (Olver et al., 1998). GGsTop was actually rationally designed to eliminate both of these adverse drug interactions. Like acivicin, GGsTop is an irreversible inhibitor of GGT but is specifically designed for added potency and GGT selectivity (Han et al., 2006, 2007). Thus far GGsTop has exhibited no reported toxicity *in vivo* and has been utilized in a renal ischemia/reperfusion injury model in rats (Yamamoto et al., 2011). We did not observe any gross toxicity in normal mice with the doses used in this study.

Interestingly, systemic delivery of GGsTop at a single time inhibited serum GGT activity in a dose dependent manner and to a greater degree than airway delivery. But GGT activity reappeared within 24 h suggesting a more rapid clearance of GGsTop from the blood and replacement with active enzyme. GGT activity in LLF was totally unaffected by systemic delivery of GGsTop. Instead, GGsTop had to be delivered thru the airway to inhibit this pool of GGT activity within the lung. This was similar to our previous experience with acivicin (Lowry et al., 2008). The basis for this is not fully known. It is possible that first pass metabolism and clearance through the liver limits drug availability in the lung. Alternatively, both GGsTop and acivicin are hydrophilic and neither appears to penetrate the lung from the blood very efficiently. Several derivatives of GGsTop have been described and hydrophobicity may be related to ability to penetrate into the lung from the blood stream and inhibit LLG GGT activity (Han et al., 2006, 2007; Joyce-Brady and Hiratake, 2011). Nonetheless, airway delivery of GGsTop was effective in inhibiting GGT activity in LLF and in serum as well. Inhibition of serum GGT activity lasted up to 24 h after inhalation. Hence, there may be an advantage to inhaled GGsTop in that the lung can function as a GGsTop reservoir for the slow delivery of drug to the periphery to maintain inhibition of systemic GGT activity and augment plasma glutathione content and antioxidant defense which is known to be depleted during the pathophysiological state of sepsis (Malmezat et al., 2000).

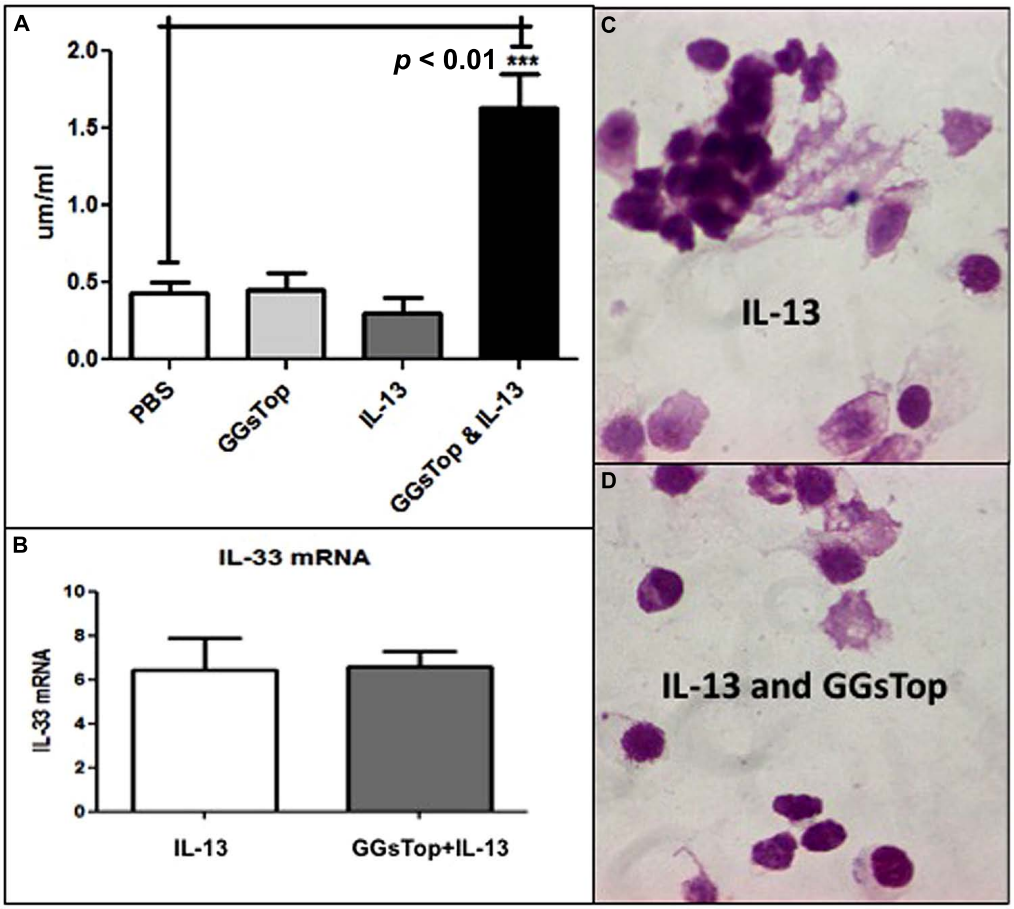

FIGURE 3 | (A) GGsTop augments LLF glutathione in the presence of IL-13. Total Glutathione concentration (μM/ml) was measured in LLF at the end of the IL-13 experiment as described in "Materials and Methods" mice with the saline treated mice as control (0.4 μM/ml, $n = 3$). The glutathione concentration was similar to that of control after treatment with GGsTop ($n = 3$) and IL-13 ($n = 3$). It was significantly increased fourfold (1.6 μM/ml) in mice treated with GGsTop along with IL-13 (***$p < 0.01$, $n = 3$). **(B–D)** The IL-13 proinflammatory response is unaffected by GGsTop as IL-33 messenger RNA expression **(B)**, assessed by qRT-PCR in total RNA isolated from lung as described in "Materials and Methods," is induced sixfold by IL-13 treatment alone ($n = 3$) and with GGsTop ($n = 3$), and an eosinophilic infiltrate predominates with IL-13 alone **(C)** and with GGsTop **(D)** as described by Lowry et al. (2008).

Lung lining fluid GGT activity was inhibited equally well by the two doses (0.5 or 5 mg/kg) of GGsTop used in our study and we used the lower dose for all subsequent experiments. This result may reflect the fact that the lung has much less GGT activity overall compared to organs like the kidney or the pancreas even though GGT activity in the LLF is concentrated sevenfold over that of the lung as a whole (Joyce-Brady et al., 1994). Relative inhibition of GGT activity in LLF was somewhat less than that of the serum. The basis for this is not yet clear and further experiments will be required to determine if the level of GGT inhibition in the lung can be optimized further. Several derivates of GGsTop are available and can be tested for differential inhibitory activity against LLF GGT (Han et al., 2007; Joyce-Brady and Hiratake, 2011).

The advantage of this IL-13 cytokine driven model of allergic airway inflammation is in the similar levels of inflammatory cells that are recruited to the lung. This permits one to focus on lung airway epithelial cells and their response to the inflammatory process (Joyce-Brady et al., 2012). Similarity of the inflammatory response after IL-13 delivery to the lung in this study is suggested by the comparable levels of induction of IL33 cytokine messenger RNA and eosinophilic infiltrate in bronchoalveolar lavage fluid compared to untreated controls in the absence or presence of GGsTop. However, that the lung responded differently after IL-13 delivery in the presence of GGsTop was shown in three ways. Methacholine-stimulated airway hyperreactivity was significantly attenuated in mice treated with IL-13 and GGsTop, whereas it was induced in mice treated with IL-13 alone. Induction of mucin gene expression, assayed as messenger RNA expression for Muc5ac and Muc5b, and mucous accumulation, visualized by PAS staining, were significantly attenuated in the lungs of mice treated with IL-13 and GGsTop, but all were induced dramatically in mice treated with IL-13 alone compared to control mice. In all of our experiments using this IL-13 model, we hypothesize that effective buffering of inflammation-derived reactive oxygen species by the augmented glutathione pool in lung lining

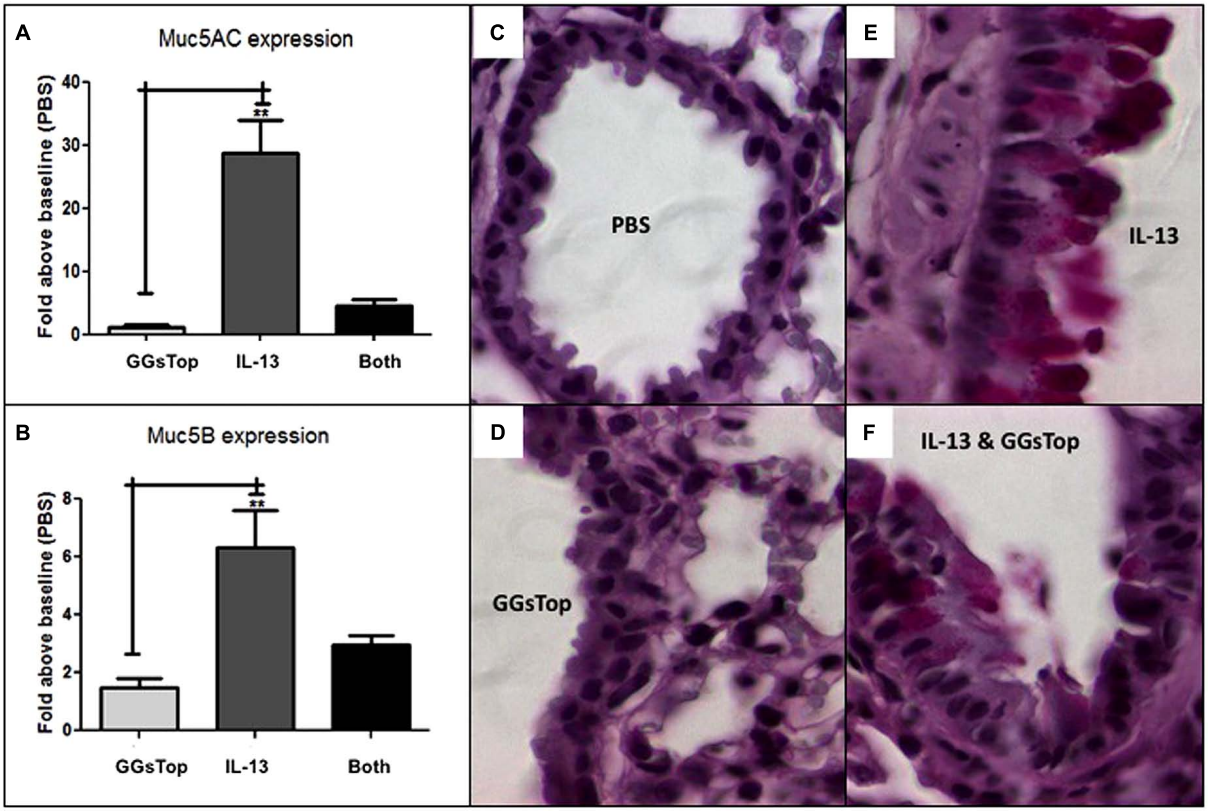

FIGURE 4 | GGsTop attenuates mucous production induced by IL-13. Compared to saline control, lung level of Muc5ac **(A)** messenger RNA expression was not affected by GGsTop alone, but it was induced 28-fold by IL-13 treatment (**$n = 3$, $p < 0.001$). When GGsTop was delivered with IL-13, this level of messenger RNA induction was decreased sevenfold. **(B)** Similarly, Muc5b messenger RNA was induced sixfold **(B)** by IL-13 treatment (**$n = 3$, $p < 0.01$) and this was decreased twofold when GGsTop was delivered with IL-13. PAS stained lung **(C–F)** shows absence of mucous in control **(C)** and GGsTop treated **(D)** lung, and a diffuse mucous signal in airway epithelial cells after IL-13 treatment **(E)** but only rare mucous signal in a few cells when GGsTop was added to IL-13 **(F)** as described by Lowry et al. (2008).

fluid protected airway epithelial cells from injury. LLF total glutathione was augmented in the IL-13 treated GGT^{enu1} mouse and in the normal mouse treated IL-13 and GGsTop, although to a lesser degree. We believe this protection preserved airway epithelial cell barrier function, prevented mucin gene induction and attenuated development of airway hyperreactivity (Lowry et al., 2008).

Other investigators have also proposed inhibiting GGT activity as a treatment for human diseases besides asthma including cancer (Hanigan, 1998), cardiovascular disease (Emdin et al., 2005), and *cis*-platinum-induced nephrotoxicity (Hanigan et al., 2001). However, augmentation of glutathione availability was not the proposed mechanism of action. Rather, it was decreased production of a pro-oxidant glutathione metabolite Cys-Gly (Emdin et al., 2005) or a toxic GGT-dependent *cis*-platinum-glutathione conjugate (Townsend and Hanigan, 2002; Townsend et al., 2003). Hanigan has even developed a novel alternative, non-toxic, and non-competitive but reversible GGT inhibitor that could also be used clinically to replace the toxicity of acivicin (King et al., 2009). Nonetheless, GGsTop, a mechanism-based and irreversible inhibitor of GGT, has been shown to be a non-toxic alternative to acivicin in a recent study where it was used to inhibit GGT activity and attenuate injury in a rat kidney ischemia/reperfusion model (Yamamoto et al., 2011). Together these studies all support the use of novel GGT inhibitors as potential therapeutics in GGT-dependent diseases. In addition, our findings may be relevant for other lung diseases that involve inflammation and perturb the extracellular LLF glutathione pool such as cystic fibrosis, adult respiratory distress syndrome, and chronic obstructive pulmonary disease (Cantin and Begin, 1991; Hull et al., 1997). Our strategy to inhibit lining fluid GGT activity is centered on the augmentation of antioxidant defense by increasing glutathione availability in the lung lining fluid, thereby preserving the barrier role of the lung epithelium and normal lung function. GGsTop and its derivatives provide a new avenue for study toward that goal.

AUTHOR CONTRIBUTIONS

Provided GGsTop and handling guidelines (Jun Hiratake); performed GGT activity analyses and interpretation (Rebecca P. Hughey, Martin Joyce-Brady); analyzed lung lining fluid glutathione (Lou Ann S. Brown, Martin Joyce-Brady); RT-PCR analyses for gene expression (Jyh-Chang Jean); analyzed methacholine-stimulated hyper-reactivity with SCIREQ

apparatus, prepared and analyzed bronchoalveolar lavage cytospins and lung histochemistry (Marina Tuzova, William W. Cruikshank, Martin Joyce-Brady); reviewed manuscript (Marina Tuzova, Jyh-Chang Jean, Rebecca P. Hughey, Lou Ann S. Brown, William W. Cruikshank, Jun Hiratake); wrote manuscript (Marina Tuzova, Martin Joyce-Brady).

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Iron and thiols as two major players in carcinogenesis: friends or foes?

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Iron is the most abundant metal in the human body and mainly works as a cofactor for proteins such as hemoglobin and various enzymes. No independent life forms on earth can survive without iron. However, excess iron is intimately associated with carcinogenesis by increasing oxidative stress via its catalytic activity to generate hydroxyl radicals. Biomolecules with redox-active sulfhydryl function(s) (thiol compounds) are necessary for the maintenance of mildly reductive cellular environments to counteract oxidative stress, and for the execution of redox reactions for metabolism and detoxification. Involvement of glutathione *S*-transferase and thioredoxin has long attracted the attention of cancer researchers. Here, I update recent findings on the involvement of iron and thiol compounds during carcinogenesis and in cancer cells. It is now recognized that the cystine/glutamate transporter (antiporter) is intimately associated with ferroptosis, an iron-dependent, non-apoptotic form of cell death, observed in cancer cells, and also with cancer stem cells; the former with transporter blockage but the latter with its stabilization. Excess iron in the presence of oxygen appears the most common known mutagen. Ironically, the persistent activation of antioxidant systems via genetic alterations in *Nrf2* and *Keap1* also contributes to carcinogenesis. Therefore, it is difficult to conclude the role of iron and thiol compounds as friends or foes, which depends on the quantity/distribution and induction/flexibility, respectively. Avoiding further mutation would be the most helpful strategy for cancer prevention, and myriad of efforts are being made to sort out the weaknesses of cancer cells.

Keywords: iron, carcinogenesis, glutathione, oxidative stress, cancer stem cell, ferroptosis, peroxiredoxins, Nrf2

INTRODUCTION

During the past 6–7 decades following World War II, the average human lifespan has been enormously extended from less than 50 years to nearly or more than 80 years in most developed countries (https://www.cia.gov/library/publications/the-world-factbook/rankorder/2102rank.html). This has been achieved at least in part by the discovery of antibiotics against bacterial infections such as tuberculosis, which had been continuously present deadly diseases until that period (Zhang, 2005). After the major human conquest over bacterial diseases, two pathologic conditions, atherosclerosis and cancer, have become the most common causes of human mortality. Atherosclerosis via the thickening of the arterial intima and rupture of atheromatous plaques causes myocardial and cerebral infarctions, which either kill the patients or dramatically decrease their quality of lives (Beckman et al., 2002). Atherosclerosis is, in a sense, an "analog" disease in that apparently nobody can escape from it (Kumar et al., 2013); rather, the speed of disease progression is much different among individuals, which is revealed with mathematical models (Hao and Friedman, 2014) (**Figure 1**). In contrast, cancer is a "digital" disease, which means that it consists of stepwise processes and is all or nothing for the generation of a malignant tumor (Weinberg, 2013) (**Figure 1**). Some patients develop secondary or even tertiary cancers after successful cancer treatments (Travis et al., 2013). In this review, I will focus on the recent advancements in the understanding of cancer regarding iron and thiol compounds that appears independent but are interdependent in many aspects.

CANCER AS A GENOMIC DISEASE

Since the discovery of oncogenes, decades of studies have revealed that cancer is basically a disease of the genomic alteration (Weinberg, 2013). Alteration of genome information after genomic damage and its inadequate repair is responsible for cancer development, and the alterations should occur in specific genes designated as oncogenes or tumor suppressor genes (**Figure 2**). There are more than 100 oncogenes identified thus far, and all of these genes are associated with cellular proliferation (Weinberg, 2013). Oncogene activation is the result of specific mutations in genes, leading to persistent activation of cellular signals toward proliferation. The genetic alterations include point mutations, gene amplifications, or gene fusions (Stratton et al., 2009; Pleasance et al., 2010). Tumor suppressor genes work as guardians of the genome by arresting the cell cycle, repairing the genome, and even inducing apoptosis after unrepairable excessive genomic injury occurs. These genes are inactivated during the carcinogenic process. It is now recognized that several (e.g., 5–8) independent or interdependent events of mixed

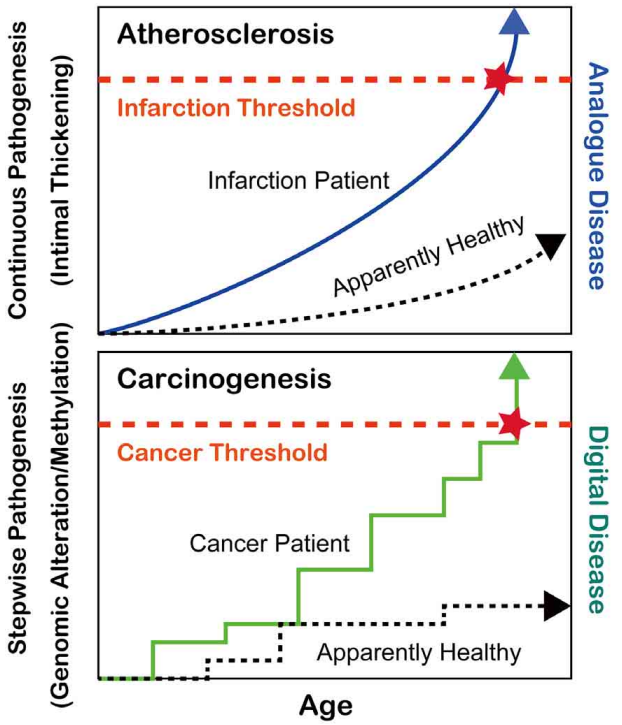

FIGURE 1 | Differences between atherosclerosis and carcinogenesis as oxidative stress-induced diseases.

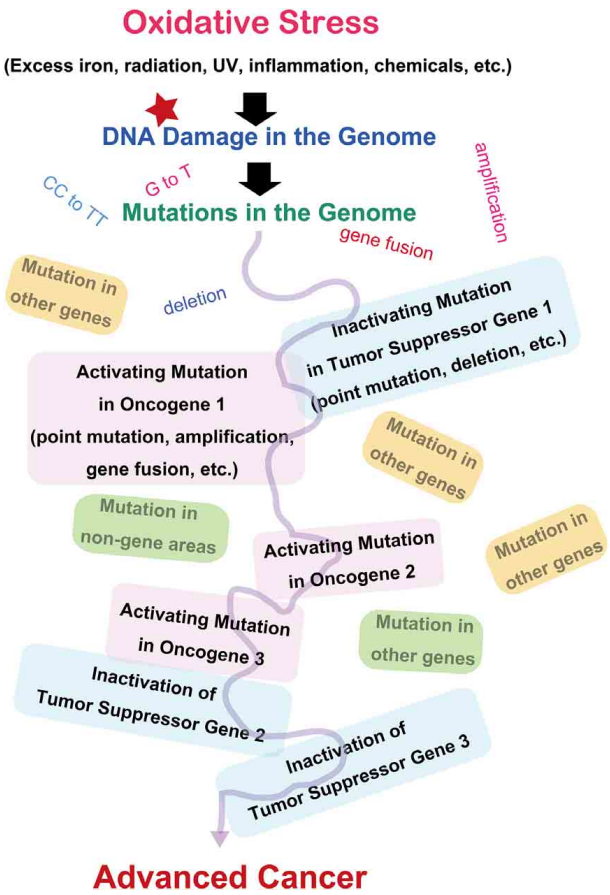

FIGURE 2 | Molecular carcinogenic processes in terms of oxidative stress.

activation of oncogenes and inactivation of tumor suppressor genes are necessary to generate a malignant tumor (Hanahan and Weinberg, 2000). Certain genetic alterations work as an instigator, facilitating the occurrence of sequential independent alterations at a significantly higher incidence (mutator phenotype) (Loeb, 2001). This is especially true of repair genes for the genome. Both alleles have to be disrupted to inactivate tumor suppressor genes. In addition, not only genetic alterations but also epigenetic changes (methylation of the promoter region) are important in halting the expression of specific genes (Feinberg et al., 2006).

As an exception, epigenetic alterations may lead to a malignant tumor in childhood cancer (Esteller and Herman, 2002). Recently, it was shown that doxycycline-controlled reprogrammable transgenic mice overexpressing the four Yamanaka factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) for induced pluripotent stem cell generation develop several cancers similar to childhood blastoma-type cancer that do not revert to normal and continue dysplastic growth, even after switching off those genes. Surprisingly, these cancers reportedly do not have major alterations of the genes, suggesting the importance of epigenetic mechanisms as well (Ohnishi et al., 2014). However, it was recently determined by next generation sequencing that genes regulating epigenetic mechanisms are one of the major targets of carcinogenesis in certain cancers such as leukemia and breast carcinoma (Smith et al., 2010; Dawson and Kouzarides, 2012). Thus, in most cases, genetic alterations regulate epigenetic mechanisms.

IRON AS A RISK OF CANCER

Iron is the most abundant metal in the human body. Approximately 4 g is present in normal adult humans. Thus, far, no life on earth can live without iron. Simultaneously, most of higher organisms, including humans, cannot survive without oxygen for 5 min. Oxygen is transported throughout the body by the heme moiety of hemoglobin, which contains as much as ∼60% of the total iron in the body (Wriggleworth and Baum, 1980; Toyokuni, 2011a). Thus, there is a natural affinity between iron and oxygen. The most important characteristic of molecular oxygen is that it is easy to be reduced via one or more of four one-electron transfer processes, ending with the formation of water (**Figure 3**). During this process, superoxide, hydrogen peroxide, and hydroxyl radicals may be generated as intermediates either via enzymatic or chemical reactions. Hydroxyl radicals are most reactive among chemical species of the biological system. Fortunately, unfavorable reactions of this kind is usually prevented via antioxidative mechanisms (Halliwell and Gutteridge, 2007; Toyokuni, 2011b).

Whereas iron is an essential component, stable Fe[III] is hardly soluble at neutral pH (10^{-17} M) (Lippard and Berg, 1994). Therefore, precise and overlapping regulatory mechanisms exist

in iron metabolism, and thus, only subtle amounts of catalytic iron are present in normal physiological conditions. Pathologic conditions, such as failure of iron regulatory sensors, repeated hemorrhage and chronic inflammation concomitant with continued parenchymal cell death, result in iron overload in the corresponding locations (Ganz, 2003), leading to oxidative damage through the Fenton reaction (Minotti and Aust, 1989; Miller et al., 1990; Halliwell and Gutteridge, 2007). Hydroxyl radicals are the most reactive species in the biological system, and the Fenton reaction *in vivo* appears to occur in the presence of catalytic ferrous iron (Samuni et al., 1983; Toyokuni, 2009), leading to the extensive scission, modification (Dizdaroglu, 1991) and cross-linking of biomolecules (Dizdaroglu, 1991). Such oxidative molecules eventually induce genomic alterations, increasing the risk of carcinogenesis. Recently, prompt formation of mono- or poly-iron $Fe^{IV} = O$ (ferryl) species was suggested at the aqueous interface (Enami et al., 2014).

Indeed, iron overload has been associated with carcinogenesis both in human and animal experiments. It is well known that there is no active excretion pathway for iron except for hemorrhage, presumably due to the supreme biological importance of iron. Intriguingly, a US epidemiological study published in 2008 reported that for peripheral arterial disease patients, phlebotomy twice a year reduced the incidence of visceral cancer by 35% and the cancer mortality by 61% in a randomized trial involving 1277 patients. In this study, iron reduction did not stop the progression of atherosclerosis, as originally intended, but did unexpectedly prevent carcinogenesis, including that of most common cancers (lung, colon, prostate, etc.) (Zacharski et al., 2007).

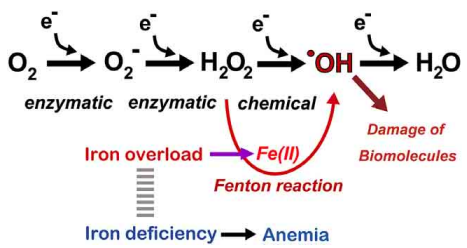

FIGURE 3 | Oxygen as a medium for electron flow and the associated role of catalytic ferrous iron (Fe[II]) toward Fenton reaction. Note that only a small fraction generates hydroxyl radicals (·OH). Refer to text and **Figure 7**.

There are several review articles published on the direct demonstration of iron overload and carcinogenesis in animal experiments (Toyokuni, 1996, 2002, 2009; Beguin et al., 2014), which I summarize as **Table 1**. Now, the role of iron in human carcinogenesis remains under intensive discussion (Cho et al., 2013; Fonseca-Nunes et al., 2014). Here, I discuss the recent results from a genuine Fenton reaction-induced carcinogenesis model generated by intraperitoneal injections of an iron chelate, ferric nitrilotriacetate (Fe-NTA), to rodents (Ebina et al., 1986; Li et al., 1987). Nitrilotriacetate (NTA) has been used as a component of detergents in Canada because of its potent chelating activity with a variety of metals, including iron (Anderson et al., 1982). First, Fe-NTA was used to load Fe[III] to transferrin, a serum iron transporting protein, in biochemistry laboratories (Pootrakul et al., 1977). Then, it was used by Awai et al. to generate an animal model of hemochromatosis (Awai et al., 1979). Of note, Okada and Midorikawa found that Fe-NTA induces renal cell carcinoma (RCC) after repeated intraperitoneal administration in wild-type rats (**Figure 4**) that were accidentally under observation for more than 1 year after the confirmation of iron accumulation in the liver (Okada and Midorikawa, 1982). Fe-NTA is soluble at neutral pH and is the most potent catalyst thus far of Fenton reaction with 3–4 free iron ligands (Toyokuni and Sagripanti, 1992, 1993).

After intraperitoneal injection, Fe-NTA is absorbed through the peritoneum into the portal vein and then enters the systemic blood flow. Thereafter, Fe-NTA is filtered through the glomeruli into the lumina of the renal proximal tubules, where Fe(III)-NTA is reduced to Fe(II)-NTA, presumably by the presence of L-cysteine (Okada et al., 1993; Okada, 1996) (**Figure 5**). The Fenton reaction indeed occurs *in vivo* in rats and mice because a variety of modified products are demonstrated in this model, including 4-hydroxy-2-nonenal (HNE) (Toyokuni et al., 1997a), HNE-modified proteins (Toyokuni et al., 1994b; Fukuda et al., 1996b), other lipid peroxidation products (Toyokuni et al., 1990; Uchida et al., 1995), 8-oxoguanine (Toyokuni et al., 1994a, 1997b), thymine-tyrosine cross-links (Toyokuni et al., 1995a) and other oxidative DNA base modifications (Toyokuni et al., 1994a).

Table 1 | Models of iron-induced carcinogenesis using wild-type animals.

Iron compounds	Administration route	Species	Induced cancer	References
Iron oxide	Inhalation	Mouse	Lung adenocarcinoma, fibrosarcoma	Campbell, 1940
Iron dextran complex	Intramuscular	Rat	Spindle cell sarcoma	Richmond, 1959
Ferric nitrilotriacetate	Intraperitoneal	Rat	Renal cell carcinoma	Ebina et al., 1986; Nishiyama et al., 1995; Tanaka et al., 2000, 2004; Akatsuka et al., 2012
Ferric nitrilotriacetate	Intraperitoneal	Mouse	Renal cell carcinoma	Li et al., 1987
Ferric saccharate	Intraperitoneal	Rat	Malignant mesothelioma	Okada et al., 1989; Hu et al., 2010
Ferric ethylene-diamine-N,N′-diacetate	Intraperitoneal	Rat	Renal cell carcinoma	Liu and Okada, 1994

The models shown above demonstrate the carcinogenicity of iron compounds in rodents.

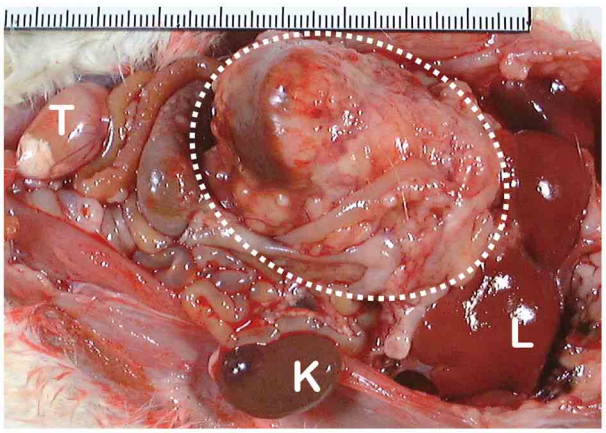

FIGURE 4 | Macroscopic appearance of ferric nitrilotriacetate (Fe-NTA)-induced renal cell carcinoma (interrupted circle; tumor diameter is more than 40 mm). K, normal kidney of the opposite side; L, liver; T, testis.

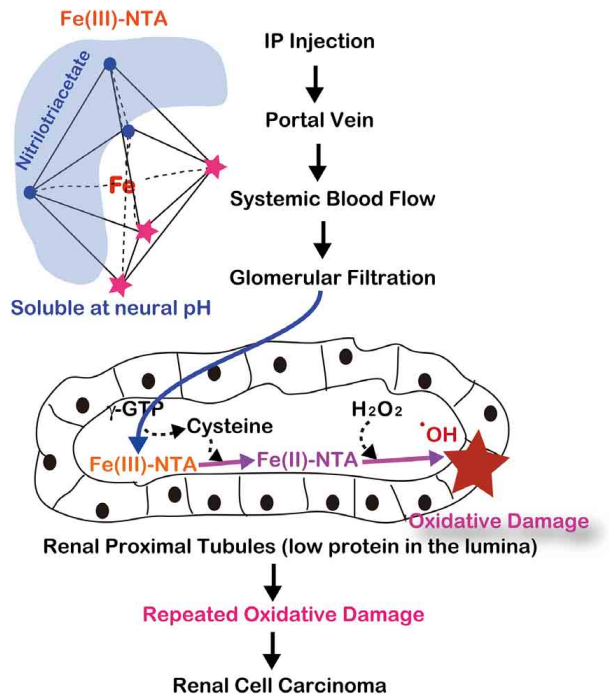

FIGURE 5 | Molecular mechanism of ferric nitrilotriacetate (Fe-NTA)-induced oxidative damage of renal proximal tubules after a single intraperitoneal injection. This depends on two distinct characteristics of renal proximal tubular lumina: paucity of antioxidattive proteins such as albumin and reductive environment through the presence of L-cysteine with GSH cycles.

DNA single- and double-stranded breaks have also been shown *in vitro* (Toyokuni and Sagripanti, 1993, 1996).

Recently, we demonstrated an abundance of Fe(II) in the lumina of renal proximal tubules after Fe-NTA injection (Mukaide et al., 2014) with a novel fluorescent probe (RhoNox-1) specific for catalytic ferrous iron (Hirayama et al., 2013). Furthermore, we showed that the induced RCCs have massive chromosomal alterations similar to those of human cancers, of which the amplification of *c-Met* (receptor for hepatocyte growth factor) and deletion of *Cdkn2a/2b* ($p16^{Ink4a}/p15^{Ink4b}$ tumor suppressor genes) are most common (Tanaka et al., 1999; Akatsuka et al., 2012). The latter occurs early in carcinogenesis (Hiroyasu et al., 2002). Considering that it is rare thus far to find massive chromosomal alterations in any other carcinogenesis model using wild-type animals, we believe that iron overload is one of the most important risk factors in human carcinogenesis as well. Of note, asbestos- (Jiang et al., 2012) and multi-walled carbon nanotube-induced (Nagai et al., 2011) mesothelial carcinogenesis in wild-type rats are the models that confer massive chromosomal alterations including homozygous deletion of *Cdkn2a/2b*. We believe that these are through local iron overload (Toyokuni, 2013b).

There are several distinct human diseases that preclude cancer via iron overload (**Table 2**). Endometriosis is defined by the presence of endometrial tissue outside of the uterine cavity and occurs in as many as 10% of women in their reproductive years. An epidemiological study revealed that ovarian endometriosis is associated with a high risk for clear cell carcinoma (Pearce et al., 2012). Because monthly menstrual hemorrhage occurs in these ectopic tissues, local iron overload is generated *in situ* (Yamaguchi et al., 2008). We recently studied endometriosis-associated ovarian clear cell carcinoma with an array-based comparative genome hybridization and found that *c-Met* (the same target gene as those of Fe-NTA-induced RCCs) is the most frequently amplified gene (Yamashita et al., 2013). Recently, it was shown with the use of knockout mice for *Cdkn2a/2b* and/or *Pten* that DNA double-stranded breaks cooperate with the loss of Ink4 and Arf (protein products from *Cdkn2a/2b* after alternative splicing) tumor suppressors to generate glioblastomas with frequent *c-Met* amplification (Camacho et al., 2014). It is remarkable that all of the necessary genomic alterations occurred in the Fe-NTA-induced RCC model of wild-type animals.

Together, these results indicate that the Fenton reaction can induce deletion/amplification mutations in target genes during carcinogenesis, presumably through DNA double-stranded breaks (**Figure 6**). Currently, next-generation sequencing studies are in progress for the above-mentioned tumors and may help determine whether iron overload can induce driver point mutations and create fusion genes through chromosomal translocations.

REGULATION OF HYDROGEN PEROXIDE VIA THIOL COMPOUNDS

Here I discuss the other partner of Fenton reaction, hydrogen peroxide. Hydrogen peroxide, a non-radical species, is a normal metabolite occurring at an approximately 10 nM intracellular concentration. Its increase in concentration may initiate Fenton reaction in the presence of catalytic Fe[II]. In the liver, which has one of the highest metabolic activities in the body, hydrogen peroxide is produced at 50 nmol/min/g tissue (Sies, 2014). In the cells and in tissues with iron overload,

Table 2 | Human carcinogenesis associated with iron overload.

	Target organ(s)	Pathogenesis	Cancer	Major genetic alterations	References
Genetic hemochromatosis	Liver	Hereditary disorder (types 1–5); excessive iron absorption	Hepatocellular carcinoma, gastric cancer, etc.		Fracanzani et al., 2001; Agudo et al., 2013
Viral hepatitis B and C	Liver	Autoimmunity-induced hepatocyte damage and iron accumulation	Hepatocellular carcinoma		Bonkovsky et al., 1997; Kato et al., 2007
Endometriosis	Ovary	Monthly menstrual hemorrhage in ectopic endometrial tissue	Clear cell carcinoma, endometrioid adenocarcinoma	Amp of *c-MET*	Pearce et al., 2012; Yamashita et al., 2013
Asbestos exposure	Mesothelium, lung	Content and adsorption of asbestos fiber; chronic inflammation by foreign body	Malignant mesothelioma; lung cancer	HD of *CDKN2A/2B*, Amp of *c-MET*	Jiang et al., 2012; Aierken et al., 2014

Amp, amplification; HD, homozygous deletion.

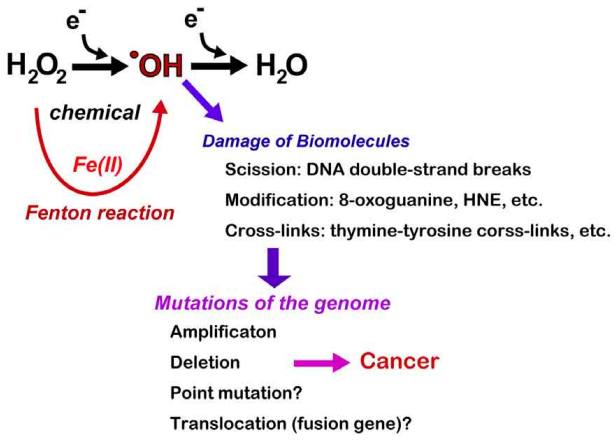

FIGURE 6 | Consequences of Fenton reaction in the genome DNA during carcinogenesis. HNE, 4-hydroxy-2-nonenal.

the regulation of the concentration and localization of hydrogen peroxide is a critical issue. It is now widely accepted that hydrogen peroxide is utilized in metabolic regulation in ways similar to diffusible gasses such as CO, NO, and H_2S (Yang et al., 2008).

A major source of hydrogen peroxide comes from the dismutation of the superoxide anion radical, which is mainly generated through NAD(P)H oxidases operated under the control of growth factors and cytokines, such as interleukin-1 and tumor necrosis factor-α (Jiang et al., 2011). This mechanism is actively used for antibacterial defense in neutrophils and macrophages during inflammation (Bedard and Krause, 2007). Another major source of hydrogen peroxide resides in the physiological mitochondrial processes through Complex I, II, and III (Cadenas and Davies, 2000).

The metabolic elimination of hydrogen peroxide includes the catalytic reaction, which is performed by catalase in peroxisomes as well as by numerous peroxidases (**Figure 7**). In addition, in tissues, hydrogen peroxide diffuses away from its source across the plasma membrane to the extracellular space, or even to adjoining cells, occurs (Giorgio et al., 2007). Various peroxidases are under the control of metabolic signals, and the most potent peroxidase is peroxiredoxins (Rhee et al., 2005). The 10^6-fold higher rate constant of the reaction of hydrogen peroxide with the cysteine thiolate in peroxiredoxins using thioredoxin as a substrate in comparison to most other deprotonated thiol compounds gives them a major role in the biological chemistry of hydrogen peroxide removal (Winterbourn, 2013). However, cysteine residues of peroxiredoxins are easily hyperoxidized to cysteine sulfinic acid, resulting in the inactivation of peroxidase activity. As a result, if this occurs, there is an accumulation of hydrogen peroxide, allowing the oxidation of specific target proteins, a phenomenon that is comparable to the opening of a gate for signaling (Wood et al., 2003). This is the molecular basis for hydrogen peroxide compartmentation in signaling (Antunes and Cadenas, 2000) (**Figure 7**). This activation is finally shut down by sulfiredoxin, which recovers hyperoxidized peroxiredoxins (Jeong et al., 2012). Glutathione peroxidases in various distinct subcellular compartments and cells play a major function in the regulation of hydrogen peroxide and lipid peroxides (Brigelius-Flohe and Maiorino, 2013). Glutathione reductase and the efflux of oxidized glutathione (GSSG) allow the maintenance of the fraction of reduced glutathione (Sies, 1999). Thioredoxin peroxidase, a selenium-dependent enzyme, also has an important role in the elimination of hydrogen peroxide (Lu and Holmgren, 2014).

Recently, hydrogen peroxide has been shown to use water channels, the aquaporins, to cross the lipid membrane more rapidly than diffusion allows (Bienert et al., 2006). Specific aquaporins promote the diffusion of hydrogen peroxide and are thus referred to as peroxiporins (Bienert et al., 2007). Aquaporin-8 can modulate the transport of hydrogen peroxide produced by NAD(P)H oxidase in leukemia cells (Bienert et al., 2007), suggesting novel targets for cancer therapy in that, in general,

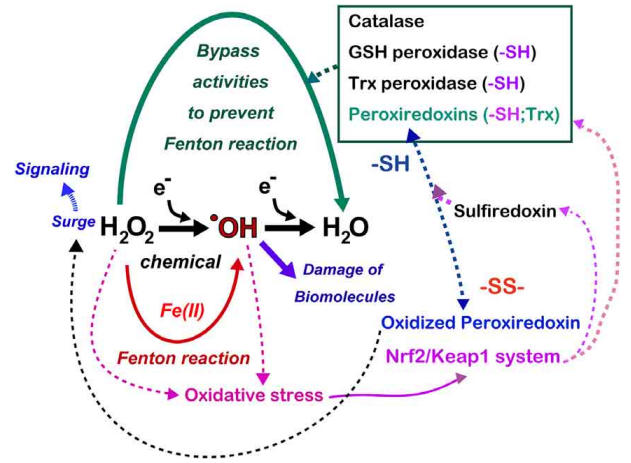

FIGURE 7 | Antagonizing role of iron and thiol compounds. Numerous overlapping mechanisms using thiol compounds exist to decompose hydrogen peroxide to water in order to bypass the generation of hydroxyl radicals. Hydrogen peroxide is now recognized as a signaling molecule whose main regulator is the peroxiredoxin/sulfiredoxin systems, which are at least partially under the control of the Nrf2/Keap1 system. Some cancers hijack Nrf2/Keap1 system with mutation in these genes, which persistently activate antioxidant systems in the cancer cells. Refer to text and **Figure 8** for details.

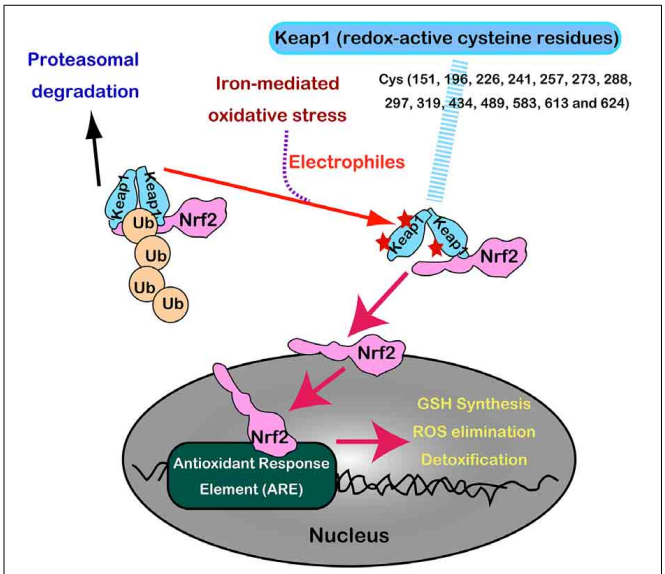

FIGURE 8 | Regulation of Nrf2 transcription machinery through oxidative stress sensor, Keap1, with numerous redox-reactive cysteine residues.

cancer cells are under persistent oxidative stress (Toyokuni et al., 1995b).

THIOL-DEPENDENT ANTIOXIDANT SYSTEMS AND CANCER

The GSH and thioredoxin systems are generally activated in cancer (Tanaka et al., 1997; Dutta et al., 2005; Nogueira and Hay, 2013; Penney and Roy, 2013; Traverso et al., 2013). Among them, there has been much interest in the overexpression of GSH *S*-transferase in rodent hepatocarcinogenesis (Hatayama et al., 1993) and in human cancers (Huang et al., 2013; Tang et al., 2013). GSH *S*-transferase pi has now been connected with peroxiredoxin-6 for its recovery of peroxidase activity (Zhou et al., 2013). Acute temporary as well as persistent overexpression of GSH *S*-transferase pi was observed during renal carcinogenesis induced by Fe-NTA (Fukuda et al., 1996a; Tanaka et al., 1998).

Nrf2 and Keap1 are now recognized as a master regulatory transcription system for antioxidant enzymes (GSH synthesis, hydrogen peroxide removal, detoxification, drug excretion, and NADPH synthesis) (Suzuki et al., 2013). Under normal conditions, Nrf2 is constitutively produced but is inactivated in the cytoplasm following its interaction with Keap1 by ubiquitination and proteasomal degradation. Keap1 is indeed a sensor molecule for oxidative stress. The multiple cysteine residues on Keap1, which are ultrasensitive to electrophiles, are critically important for the binding with Nrf2 (Itoh et al., 1997, 1999; Mitsuishi et al., 2012) (**Figure 8**).

With the aid of next generation sequencing, it has become clear that Nrf2 is consistently activated in certain cancers with various mutations. Mutually exclusive Nrf2 mutations or Keap1 mutations are observed in cancers for Nrf2 to be localized in the nucleus (Mitsuishi et al., 2012). Sulfiredoxin is also under the transcriptional regulation of Nrf2 (Jeong et al., 2012). Thus, cancer cells have hijacked this system, making them consistently more resistant to oxidative stress (**Figure 7**). Recently, peroxiredoxin 1, 4, and 6 were shown either to enhance tumor progression or to promote metastasis (Ishii et al., 2012).

CANCER STEM CELLS AND THIOL METABOLISM

Stem cells are defined as undifferentiated immature cells that, upon certain stimuli or signaling, differentiate into a planned type of mature cell(s). Cancer stem cells represent a distinct subset, namely, cells that have acquired all the necessary genetic and epigenetic alterations but are usually quiescent and divide only if necessary (Holland et al., 2013). This feature is in contrast to the cancer tissue as a whole, which is exposed to persistent oxidative stress (Toyokuni et al., 1995b). In this sense, cancer stem cells constitute small heavenly territories in cancer tissue. In transplant experiments, theoretically, even a single cancer stem cell can generate a large tumor. Thus, the existence of cancer stem cells has been used to explain chemotherapy-resistance in such a dormant state. These cancer stem cells are more resistant than non-stem cancer cells to cytotoxic chemicals.

Recently, Hideyuki Saya's group reported that cancer stem cells in certain cancers present CD44 variant 8-10 (CD44v) isoform on the plasma membrane, which stabilizes the cystine/glutamate transporter (antiporter; xCT), leading to increased GSH (Ishimoto et al., 2011). This may at least partially explain the robustness of cancer cell defenses against oxidative stress, including those involved in chemotherapy-resistance. There is already an xCT antagonist, sulfasalazine, and clinical trials are underway in advanced gastric cancer in Japan (**Figure 9A**).

FERROPTOSIS

Recently, a different type of cell death other than necrosis, apoptosis, or autophagy was reported in cancer cells. The oncogenic RAS-selective lethal small molecule erastin triggers a unique iron-dependent form of non-apoptotic cell death called ferroptosis (Dixon et al., 2012). Similar to glutamate, erastin inhibits cystine uptake by the cystine/glutamate antiporter, inducing a void of antioxidant defenses, resulting in iron-dependent oxidative stress. Interestingly, this type of cell death is inhibited by an iron chelator, deferoxamine, which removes cellular iron (**Figure 9B**). Deferoxamine functions in clear contrast to NTA in that it blocks all 6 ligands of iron (Toyokuni and Sagripanti, 1992). The authors suggest that one of more yet unidentified iron-dependent enzymes are functioning as a core lethal mechanism for ferroptosis (Dixon et al., 2012).

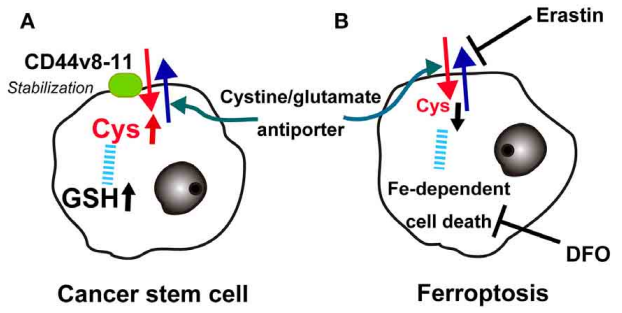

FIGURE 9 | Key role of the cystine/glutamate antiporter in cancer cells. **(A)** Overexpression of CD44v(8-11) stabilizes the cystine/glutamate antiporter to increase cysteine and glutathione (GSH) in cancer stem cells. **(B)** Conversely, erastin blocks the cystine/glutamate antiporter, lowers cysteine and finally induces iron-dependent cancer cell death (ferroptosis), which can be blocked with deferoxamine (DFO).

FUTURE CANCER PREVENTION AND CANCER THERAPEUTICS

It is generally accepted that chronic oxidative stress via excess iron leads to carcinogenesis, presumably through hydroxyl radicals, and that most of the defense system is associated with thiol compounds whereas iron and thiol compounds are apparently essential elements. These two are antagonistic in abundance, and their characteristics are distinct (**Table 3**).

For cancer prevention, we recognize that cancers of different tissues are completely different diseases. Specific risks are present for each type of cancer, and we have to decrease specific risks as early as possible in our lives. For example, asbestos is a definite carcinogen, causing malignant mesothelioma, and smoking increases the risk of more than 20 different cancers, including laryngeal and lung cancers (Toyokuni, 2013a). Today we demand practical methods for prevention of overall cancer. Antioxidant systems, if deficient, have to be supplemented.

Table 3 | Antagonizing roles of iron and thiols.

	Iron	Thiol compounds
Transition	Fe(II), transport across membrane, cytosol/Fe(III), extracellular)	-SH(reduced)/-SS-(oxidized)
Reaction	Catalytic Fe(II): Fenton reaction in the presence of hydrogen peroxide; oxidative damage by hydroxyl radicals, irreversible but usually limited with various preventive mechanisms	Common; non-destructive; redox regulation; usually reversible with reducing enzymes (**Figure 7**); free form, H_2S, SH^- (Nishida et al., 2012)
Biological significance	Cofactor of proteins (heme)/enzymes (catalytic site); stored as ferritin; oxygen transport and storage	Formation of mildly reductive intracellular environments; mostly present as cysteine residue in peptides (GSH) or proteins (thioredoxin, metallothionein, etc.); cystine when oxidized; redox signal
Metabolism	Slow; essential nutrient; nearly closed system (whole 4 g in adults; 1 mg in and out daily); no excretion pathway except bleeding (hemoglobin); transported by transferrin and its receptor system	Fast; cysteine is synthesized from methionine (essential amino acid)
Regulation	Iron transporters; HFE, hepcidin, IRP-1 and -2 (posttranscriptional) (Hentze and Kuhn, 1996), etc.	Transcription factors: Nrf2/Keap1, AP-1, NF-κB (Schenk et al., 1994; Jeong et al., 2012), etc.
Deficiency/excess/toxicity	Deficiency causes anemia; excess leads to oxidative tissue damage and sometimes carcinogenesis	Toxicity known for certain thiol compounds to initiate Fenton-like reaction (Munday, 1989); persistent activation of the associated systems is observed in cancer and its stem cell (refer to text for details)
Molecular affinity	O_2, CO, NO; transferrin, siderophore (Devireddy et al., 2010); mitoferrin (Shaw et al., 2006), frataxin (Schmucker et al., 2011), Fe-S cluster (Hentze et al., 2010)	Electrophiles (Dennehy et al., 2006), toxic metals (Hg, Cd, Pb, and As) (Quig, 1998), 8-nitro-cGMP (Nishida et al., 2012)

However, thus far, antioxidant supplementation has not officially been recommended based on the results of epidemiological studies (Bjelakovic et al., 2004, 2007). Excessive supplementation with β-carotene even increased the risk of lung cancer in smokers (Albanes et al., 1996). Here appropriate iron reduction via blood donation or phlebotomy may be a potential method of cancer prevention. Among the three components of iron, oxygen, and thiol compounds, we can modify only iron status after all. Humans live much longer than they did 70 years ago, following the conquest of major infectious diseases. After reaching middle age, iron is found in excess, especially in men of well-developed countries, because there is no other way to excrete iron (Toyokuni, 2011a). This theory requires further epidemiological studies and clinical trials for demonstration. Reportedly, cancer may hijack cytokine systems (e.g., SMAD4) via mutation to collect iron for proliferation (Wang et al., 2005). Notably, there is an opposing report that iron deficiency accelerates Helicobacter pylori-induced carcinogenesis in rodents and humans (Noto et al., 2013). Iron deficiency appears to enhance the virulence of Helicobacter pylori, which definitely requires iron to live. This fact needs to be further discussed. Iron deficiency should be avoided because it causes anemia (hemoglobin) and muscle weakness (myoglobin).

Currently, antibody therapies (Scott et al., 2012) and small-molecular-weight kinase inhibitors (Fabbro et al., 2012) are popular and work well as individualized therapies for specific cancers. However, the drawbacks of these therapies include the acquisition of resistance and high medical costs. As an alternative approach, some scientists are already thinking of attacking the Achilles' heel of cancer. As foreseen from the presence of ferroptosis, cancers in general accumulate iron for proliferation, which may allow the abundance of catalytic iron in the cytoplasm. Indeed, hydrogen peroxide is not the only molecule to induce the Fenton reaction. Ascorbate (vitamin C) and L-cysteine as reducing agents can also initiate the Fenton reaction in the presence of catalytic Fe(III) (Toyokuni and Sagripanti, 1996). In light of this, high-dose ascorbate therapy is being tested in a clinical trial with a standard regimen of chemotherapy because ascorbate has long been proven to be a safe drug in humans (Welsh et al., 2013).

EPILOG

Iron and sulfur are essential for life despite their presence in small amounts. Excessive iron cause oxidative damage in the genome, which can be a basis of somatic mutational evolution in search of resistance against oxidative stress and cellular proliferation. Apparently, iron and thiol compounds are antagonistic toward oxidative stress, but even thiol compounds can be our foes in cancer. Therefore, it is difficult to conclude the role of iron and thiol compounds as friends or foes, which depends on the quantity/distribution and induction/flexibility, respectively. Avoiding further mutation would be the most helpful strategy for cancer prevention, and myriad of efforts are being made to sort out the weaknesses of cancer cells.

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Glutathione *S*-conjugates as prodrugs to target drug-resistant tumors

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Living organisms are continuously exposed to xenobiotics. The major phase of enzymatic detoxification in many species is the conjugation of activated xenobiotics to reduced glutathione (GSH) catalyzed by the glutathione-*S*-transferase (GST). It has been reported that some compounds, once transformed into glutathione *S*-conjugates, enter the mercapturic acid pathway whose end products are highly reactive and toxic for the cell responsible for their production. The cytotoxicity of these GSH conjugates depends essentially on GST and gamma-glutamyl transferases (γGT), the enzymes which initiate the mercapturic acid synthesis pathway. Numerous studies support the view that the expression of GST and γGT in cancer cells represents an important factor in the appearance of a more aggressive and resistant phenotype. High levels of tumor GST and γGT expression were employed to selectively target tumor with GST- or γGT-activated drugs. This strategy, explored over the last two decades, has recently been successful using GST-activated nitrogen mustard (TLK286) and γGT-activated arsenic-based (GSAO and Darinaparsin) prodrugs confirming the potential of GSH-conjugates as anticancer drugs.

Keywords: glutathione *S*-conjugate, prodrug, cancer, drug resistance, glutathione transferase, gamma-glutamyl transferase

INTRODUCTION

Glutathione (GSH) plays a myriad of roles in the body. It is a major cellular antioxidant, involved in defense against oxidative stress and redox signaling. GSH also modulates cell proliferation, apoptosis, immune function, and fibrogenesis (Lu, 2013). In cancer cells, GSH and enzymes of the mercapturic acid pathway play a role in resistance to many chemotherapeutic drugs. However, not all xenobiotics (including drugs) are conjugated/inactivated by this pathway (Commandeur et al., 1995); some are in fact activated into cytotoxic compounds. This review explores the importance of the mercapturic acid pathway and the potential of intra-tumor activation of old and recently discovered chemotherapeutics. The expression patterns of participating enzymes of the mercapturic acid pathway could theoretically be employed to drive the activation of such compounds within the tumor.

The mercapturic acid pathway (**Figure 1**) involves the conjugation of the tripeptide, GSH, to xenobiotics (including drugs) to render them more hydrophilic and facilitate their elimination. Although some spontaneous reactions could occur, the cytosolic glutathione transferases (GST) catalyze the nucleophilic conjugation of GSH with a wide spectrum of electrophiles (Armstrong, 1997). Being the first step in the metabolism and eventual removal of the drug from the body, GST is associated with chemotherapeutic resistance since exposure to these drugs is often associated with induction of GST, especially GST P1-1 (Dahllof et al., 1987; Hamada et al., 1994; Hao et al., 1994). The GSH-conjugated compounds generated are then actively pumped out of the cell by numerous members of the MRP/ABCC family, which appear to have broad and partially overlapping substrate specificity (Ballatori et al., 2009). At the cell surface, the first step in the catabolism of GSH-conjugates is catalyzed by the membrane-bound gamma-glutamyl transferase (γGT) which removes the GSH γ-glutamyl group and transfers it to appropriate acceptors. Similar to what has been described for GST, γGT is also considered to be part of a resistance phenotype. The main reason for this is the role that γGT plays in maintaining appropriate GSH levels in the cells for xenobiotic detoxification by conjugation (Pompella et al., 2006). The newly formed cysteinylglycine *S*-conjugates are further processed by dipeptidases/aminopeptidases to remove the glycyl group and produce cysteine *S*-conjugates. These compounds then re-enter the cell via various transporters including organic anion transport polypeptides and cystine/cysteine importers (Hinchman et al., 1998; Budy et al., 2006; Dilda et al., 2008, 2009; Garnier et al., 2014). In the cytosol, *N*-acetyl transferases create mercapturic acid versions of the xenobiotics which are generally more polar and more water soluble than the parental compound. At this point, the compounds are generally non-toxic and excreted from the body through bile or urine. Alternatively, instead of acetylation, some compounds can be converted by cysteine *S*-conjugate β-lyase to produce an unstable and highly reactive thiol.

Abbreviations: 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine (6-mercaptopurine and 6-thioguanine); CAO, 4-[*N*-(*S*-cysteinylacetyl)amino]phenylarsonous acid; cAVTP, *cis*-6-(2-acetylvinylthio)-purine; CPIC, *p*-chlorophenyl isocyanate; DMAC, dimethylarsino-cysteine; DMACG, dimethylarsino-cysteinylglycine; EGFR, epidermal growth factor receptor; GSH, glutathione; GCAO, 4-[*N*-(*S*-cysteinylglycyl-acetyl) amino] phenylarsonous acid; γGT, gamma-glutamyl transferase; GSAO, [4-(*N*-(*S*-glutathionylacetyl) amino)phenylarsonous acid]; GS-cAVTP, glutathione *s-cis*-6-(2-acetylvinylthio)-purine; Glu, glutamate; Gly, glycine; GS-tAVTG, glutathione *s-trans*-6-(2-acetylvinylthio)-guanine; PENAO, 4-(*N*-(*S*-penicillaminylacetyl)-amino)phenylarsonous acid; SCPG, *S*-(*N*-*p*-chlorophenylcarbamoyl) glutathione; tAVTG, *trans*-6-(2-acetylvinylthio)-guanine.

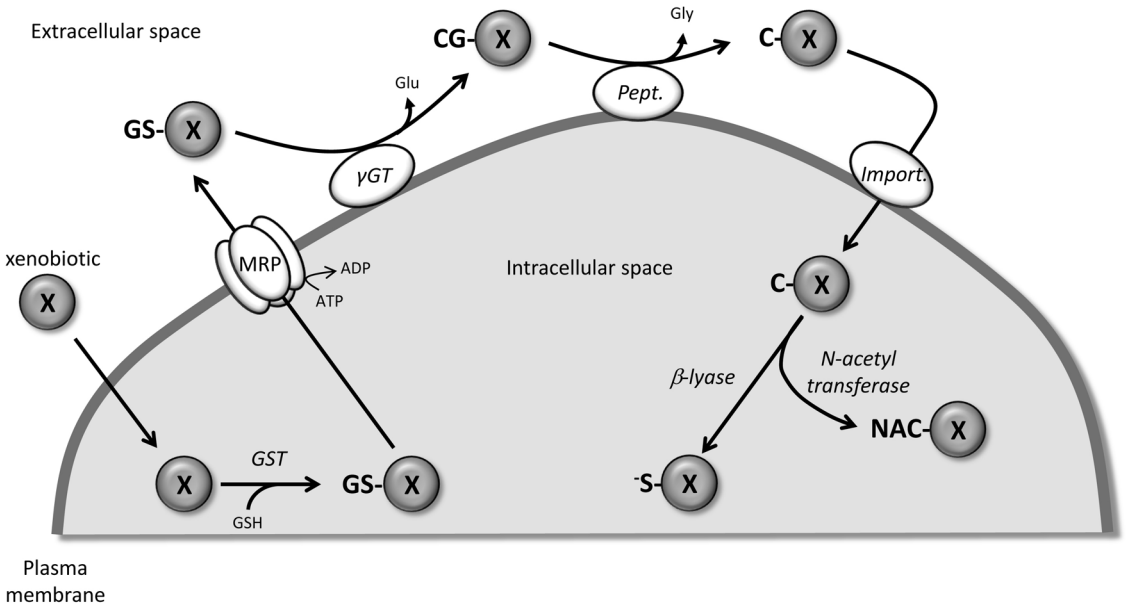

FIGURE 1 | Detoxification of xenobiotic through the mercapturic acid synthesis pathway. X, xenobiotic; GS-X, GSH-conjugated version of X; CG-X, cysteinylglycyl-X; C-X, cysteinyl-X; NAC-X, mercapturic acid version of X; $^{-}$S-X, highly reactive thiol version of X; GST, glutathione *S*-transferase, γGT, gamma-glutamyl transferase; Pept., peptidase; Import., peptide transporter or cystine/cysteine importer.

There are several examples of drugs becoming toxic/bioactive following their conjugation with GSH. Trichloroethylene is metabolized to form [*S*-(1,2-dichlorovinyl)GSH], which is the first step in the GSH metabolism pathway. *In vitro* (Lash et al., 1986; Chen et al., 1990; Cummings and Lash, 2000) and *in vivo* (Terracini and Parker, 1965; Elfarra et al., 1986), the downstream metabolites of GSH *S*-dichlorovinyl, such as *N*-acetyl-*S*-(1,2-dichlorvinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-L-cysteine, induce toxicity towards the cells responsible for their production (Spencer and Schaumburg, 1985; Patel et al., 1993). Consistent with what was observed with trichloroethylene, the GSH-*S*-conjugate of 4-hydroxynonenal (HNE) causes a loss in cell viability in γGT-expressing cells (Enoiu et al., 2002). This effect was attributed to the cysteinylglycine *S*-conjugate of HNE (Calonghi et al., 2002; Cerbone et al., 2007; Pettazzoni et al., 2011).

The expression of several enzymes involved in the mercapturic acid pathway (notably GST and γGT) is induced in cancer cells and particularly drug resistant cancer cells (**Table 1**). The specific expression of these enzymes involved in the local production of cytotoxic/bioactive drugs has been exploited to design and develop various types of anticancer prodrugs. This review describes the enzymes involved in drug activation in the order they participate along the mercapturic acid pathway, GST, γGT, and β-lyase. The potential to exploit the induced expression of these enzymes as predictive markers and the opportunity this presents for drug design is discussed.

BIOACTIVATION OF CANCER COMPOUNDS BY GST

Glutathione transferases (EC 2.5.1.18) are a superfamily of dimeric detoxification enzymes which contribute to the cellular biotransformation of electrophilic compounds (Mannervik et al., 2005). They provide protection against genotoxic and carcinogenic effects of numerous substances of both xenobiotic and endogenous origins. The essential role of GST is to catalyze the conjugation of GSH with a wide variety of compounds, including drugs, resulting in the formation of the corresponding GSH-conjugates and subsequently facilitate their clearance from the body. In humans, various isoforms of GST are present in virtually all tissues with the liver exhibiting the highest cytosolic GST activity level followed by kidney, lung, and intestine (Pacifici et al., 1988).

GST AND CANCER

Although GST detoxifying activity protects cells from endogenous toxic products, it also blunts the effectiveness of certain anticancer drugs (O'Brien and Tew, 1996). GST and GSH are frequently elevated in many tumors relative to surrounding healthy tissue (Howie et al., 1990). Isoenzyme GST P1-1 is notably induced in lung, colon, and stomach cancers and was found to be implicated in cellular resistance to chemotherapeutic agents (Dahllof et al., 1987; Hamada et al., 1994; Hao et al., 1994).

The polymorphism in GSTM1 and GSTT1 genes, leads to complete lack of activity of their corresponding enzymes and is responsible for poor elimination of carcinogenic substances which are potential sources of reactive oxygen species (Rebbeck, 1997; Kumar et al., 2011). This polymorphism has been associated with increased risk of benign prostatic hyperplasia and prostate cancer (Rebbeck et al., 1999) and can be a useful biomarker to identify patients at higher risk for fatal prostate cancer (Agalliu et al., 2006; Liu et al., 2013). In prostate, but also breast, endometrial and hepatocellular carcinomas, another early tumor marker is GSTP1 promoter methylation which is detected at various percentages of clinical samples (Esteller et al., 1998; Zhong et al.,

Table 1 | Activity and expression levels of glutathione-*S*-transferase and gamma-glutamyl transferase in tumor tissues.

Tumor	Glutathione *S*-transferase		Gamma-glutamyl transferase	
	Activity or expression vs. normal tissue	**Reference**	**Expression in tumor tissue**	**Reference**
Liver	↓ 0.50 ↓ Pol. M1, T1	Howie et al. (1990) Song et al. (2012)	++	Tsutsumi et al. (1996), Hanigan et al. (1999a)
Kidney	↓ 0.27–0.44 ↓ Pol. P1, M1, T1	Di Ilio et al. (1987), Wang et al. (1989) Ahmad et al. (2012)	++ ↑ vs. normal tissue (activity)	Hanigan et al. (1999a) Arai et al. (1995)
Esophagus	↑ 5.75 ↓ Expression P1	Tsuchida et al. (1989) Wang et al. (2010)	+ ↑ vs. normal tissue (expression and activity)	Hanigan et al. (1999a) Fiala et al. (1980)
Stomach	↑ 1.36–1.66	Howie et al. (1990), Peters et al. (1990)	±	Hanigan et al. (1999a)
Colon	↑ 1.37–4.3	Tsuchida et al. (1989), Moorghen et al. (1991)	+ ↑ vs. normal tissue (expression and activity)	Hanigan et al. (1999a) Fiala et al. (1980), Murata et al. (1997)
Pancreas	↓ Pol. M1, T1	Fan et al. (2013)	+	Hanigan et al. (1999a), Ramsay et al. (2014)
Lung	↑ 1.83–2.63	Di Ilio et al. (1987), Howie et al. (1990)	± ↑ vs. normal tissue (expression)	Hanigan et al. (1999a) Tateishi et al. (1976), Dempo et al. (1981), Blair et al. (1997)
Thyroid	↓ Pol. P1, M1, T1	Gaspar et al. (2004), Li et al. (2012)	+	Hanigan et al. (1999a)
Breast	→ 0.85–1.33 ↓ Pol., methyl. P1	Forrester et al. (1990), Howie et al. (1990) Saxena et al. (2012)	++ ↑ vs. normal tissue (expression and activity)	Durham et al. (1997), Hanigan et al. (1999a), Fiala et al. (1980), Bard et al. (1986)
Ovary	↑ Expression	Hetland et al. (2012)	+	Paolicchi et al. (1996), Hanigan et al. (1999a, 1994), Grimm et al. (2013)
Prostate	↓ Pol. P1, M1, T1 ↓ Methyl. P1	Moskaluk et al. (1997), Rebbeck et al. (1999) Esteller et al. (1998)	++ ↑ vs. normal tissue (expression and activity)	Hanigan et al. (1999a) Fiala et al. (1980)
Urinary bladder	↑ 5.17 → Expression	Lafuente et al. (1990) Oğuztüzün et al. (2011)	↑ vs normal tissue (expression and activity)	Fiala et al. (1980)

For GST, arrows indicate if the measured activity or expression is increased (↑), decreased (↓) or stable (→) in comparison with normal tissue. Values are the ratio of GST activity in tumor tissue with GST activity in normal tissue. Pol. refers to polymorphism of GST genes responsible for the loss of activity of GST P1 (P1), GST M1 (M1), or GST T1 (T1) isoenzymes. Methyl. refers to DNA methylation in the promoter region of GSTP1 gene responsible for epigenetic silencing of this isoenzyme. For gamma-glutamyl transferase, arrows indicate that the measured activity or expression is increased (↑) in comparison with normal tissue. The signs + and ± indicate the level of γGT expression in tumor tissues measured by immunohistochemistry.

2002; Chan et al., 2005; Lee, 2007; Yoon et al., 2012). The epigenetic silencing of GSTP1 provides a mechanism of resistance which makes tumors with GSTP1 promoter methylation bad candidates for the GST-activated anti-cancer prodrugs presented in this review. However, recent studies demonstrated that it is possible to re-sensitize tumors using a combination of molecules specifically reverting the aberrant DNA methylation in cancer cells (through drugs inhibiting DNA methyltransferase activity) and cytotoxic drugs (Plumb et al., 2000; Cheng et al., 2003; Sabatino et al., 2013).

GST ANTICANCER PRODRUGS

An approach to solving drug resistance due to over expression of GST is to design specific GST inhibitors (Ruzza et al., 2009). Alternatively, GST overexpression offers the opportunity to target resistant tumors with GST-activated prodrugs. This type of compound undergoes GST-catalyzed breakdown to release locally cytotoxic metabolites and thereby attenuate off target adverse side effects. Amongst these prodrugs, two categories can be distinguished.

The first category consists of prodrugs containing a GSH or GSH-like structure for which a β-elimination reaction, catalyzed by GST, releases a cytotoxic compound. This includes compounds such as nitrogen mustards, which have the property to alkylate DNA after further transformation: TLK286 (canfosfamide, TELCYTA^{TM}) and analogs (Lyttle et al., 1994; Satyam et al., 1996; Dourado et al., 2013; **Figure 2A**). TLK286 showed interesting *in vitro* and *in vivo* antiproliferative activity on cells with high GST P1-1 expression as a result of selection for doxorubicin, cyclophosphamide, and platinum resistance (Morgan et al., 1998; Townsend et al., 2002). In murine xenografts, tumor growth inhibition, or regression in response to TLK286 was positively correlated with the level of GST P1-1 expression (Morgan et al., 1998). Several clinical trials demonstrated that TLK286 was active and safe to use in combination treatment regimens with standard chemotherapeutic agents, including platinums, taxanes, and anthracyclines. Noteworthy, clinical efficacy was observed with both relapsed patients with ovarian and non-small cell lung cancers, and in the first-line treatment setting in non-small cell lung cancer patients (Sequist et al., 2009; Vergote et al., 2010).

The second category regroups prodrugs non-structurally related to GSH. However, their activation by GST involves the production of an intermediate GSH-conjugate.

– In this category, *cis*-6-(2-acetylvinylthio)-purine (cAVTP) and *trans*-6-(2-acetylvinylthio)-guanine (tAVTG) contain active thiopurines (6-mercaptopurine and 6-thioguanine, respectively) liberated intracellularly after GSH-conjugation catalyzed by GST M1-1, M2-2, and A4-4, but not GST P1-1 (Gunnarsdottir et al., 2002; Eklund et al., 2007; **Figure 2B**). Both compounds exhibited a remarkable growth-inhibitory activity towards leukemic and melanoma cells (Gunnarsdottir and Elfarra, 2004). *In vivo*, on the contrary to what was observed following the administration of bioactive end products (6-mercaptopurine and 6-thioguanine), no reduction of circulating white blood cells was observed upon

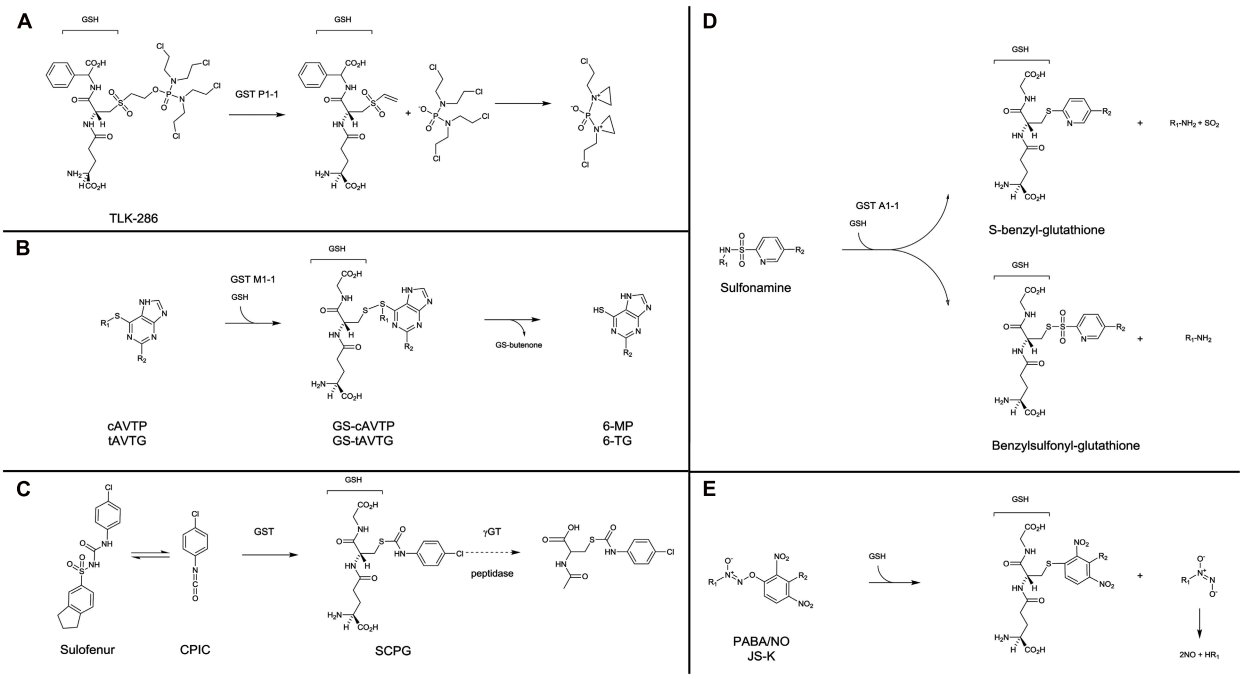

FIGURE 2 | Mechanisms of activation of GST prodrugs. TLK286 **(A)** has a GSH-like moiety in its structure, in contrast, all other presented drugs are conjugated with GSH during their process of activation by GST. **(B)** for cAVTP, GS-cAVTP, and 6-MP, R_1: *cis*-butenone; R_2: H. For tAVTG, GS-tAVTG and 6-TG, R_1: *trans*-butenone; R_2: NH_2. **(D)** for sulfonamine compounds, R_1: bombesin or aryl derivatives; R_2: CN or CF_3. **(E)** for PABA/NO, R_1: NMe2; R_2: 4-carboxy-*N*-methylanilinyl. For JS-K, R_1: 1-carboxyethyl-4 piperazinyl, R_2: H.

administration of cAVTP and tAVTG (Gunnarsdottir et al., 2002).

– Using a similar strategy, exocyclic enones displaying antitumor activity were produced by a GSH-dependent reaction catalyzed by GST (Hamilton et al., 2002, 2003). COMC-6 (2-crotonyloxymethyl-2-cyclohexenone) is a potent antitumor agent against both murine and human tumors in culture and in tumor-bearing mice (Aghil et al., 1992).
– Diarylsulfonylureas are a class of antitumor agent with interesting therapeutic efficacy against a wide range of cancers (Howbert et al., 1990; Mohamadi et al., 1992). Sulofenur [(*N*-(5-indanesulfonyl)-*N*′-(4-chlorophenyl)urea, LY18664], the prototypic member of this class (**Figure 2C**), was evaluated in clinical trials on a variety of patients with kidney (Mahjoubi et al., 1993), ovary (O'Brien et al., 1992), breast (Talbot et al., 1993), lung (Munshi et al., 1993), and stomach (Kamthan et al., 1992) cancers. It appeared that sulofenur can undergo metabolic biotransformation to yield the GSH-conjugate of *p*-chlorophenyl isocyanate [*S*-(*N*-*p*-chlorophenylcarbamoyl) GSH, SCPG; Jochheim et al., 2002]. Whether a direct involvement of GST is necessary or not in the production of this GSH-conjugate remains unclear. However, it was clearly demonstrated that SCPG is further processed through the mercapturic acid pathway to the corresponding N-acetylcysteine conjugate [*N*-acetyl-*S*-(*p*-chlorophenylcarbamoyl)cysteine, NACC], which possesses comparable anticancer activity to the parent compound. The GSH- and cysteine-conjugates produced are susceptible to thiol exchange reactions and may act as carbamoylating agents towards biomacromolecules (Day et al., 1996; Guan et al., 2002). NACC, whose mechanism of action remains unclear, demonstrated selective anticancer activity, and low toxicity, which make NACC and its analogs promising anticancer agents (Chen et al., 2011).
– Another approach was to use the sulphonamidase activity of GST. GST catalyzes the GSH-mediated hydrolysis of sulfonamide bonds, releasing GST competitive inhibitors such as benzylsulfonyl-GSH or *S*-benzyl-GSH (Koeplinger et al., 1999; Zhao et al., 1999; Axarli et al., 2009; **Figure 2D**).
– Finally, electrophilic diazenium diolates were designed as prodrugs for spontaneous nitric oxide (NO) release at physiological pH, after a reaction with GSH catalyzed by GST. Essentially two compounds were studied. PABA/NO [(O^2-(2,4-dinitro-5-(*N*-methyl-*N*-4 carboxyphenylamino)phenyl) 1-*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate; Findlay et al., 2004] and JS-K [O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl]diazen-1-ium-1,2-diolate; Shami et al., 2003] were selectively activated by GST M1 and GST A1, respectively, and produced abundant NO in the tumor and contributed to chemotherapy by GSH consumption, DNA synthesis inhibition and inhibition of enzymes responsible for cellular damage repair (**Figure 2E**). PABA/NO and JS-K demonstrated antitumor activity in mice bearing human ovarian carcinoma (Findlay et al., 2004) and prostate carcinoma (Laschak et al., 2012) or multiple myeloma (Kiziltepe et al., 2007), respectively. Recently, new generations of more stable and potent GST-activated NO prodrugs have been developed (Liu et al., 2012; Fu et al., 2013).

GLUTATHIONE *S*-DERIVATIVES ACTIVATED BY γGT AND PEPTIDASES

Gamma-glutamyl transferase (EC2.3.2.2) catalyzes the transpeptidation and hydrolysis of the γ-glutamyl group of GSH and related compounds to an acceptor molecule, including water, amino acids, and peptides. It is found on the plasma membrane, facing extracellularly, playing an essential role in the maintenance of intracellular cysteine (Moriarty-Craige and Jones, 2004). Considering GSH's important role as a cellular antioxidant, γGT has traditionally been considered a component of the cell's oxidative stress defenses. However, the range of γGT substrates has expanded considerably, now including a range of GSH conjugates, leukotriene C4 (LTC4), *S*-nitroso-GSH and GSH adducts of xenobiotics, suggesting diverse roles for γGT.

High expression of γGT is commonly found on cells involved in transport, on the luminal surface of secretory and absorptive cells. Its highest expression is on the luminal surface of the proximal tubules in the kidney, whilst the bile ducts, bile canaliculi, and endothelial cells of the nervous system capillaries also have high expression (Shiozawa et al., 1989; Hanigan and Frierson, 1996).

Oxidative stress has been shown to induce γGT expression (Kugelman et al., 1994; Knickelbein et al., 1996; Liu et al., 1998; Borud et al., 2000; Roomi et al., 2006). High γGT expression protects melanoma cells from hydrogen peroxide or ascorbic acid induced oxidative stress (Giommarelli et al., 2008). It also allows the cells to maintain their intracellular GSH levels and subsequently respond to oxidative stress. Conversely, γGT has been demonstrated to have pro-oxidant effects. Combined with metal ions (iron or copper), γGT can induce lipid peroxidation (Stark et al., 1993; Stark and Glass, 1997). γGT has been linked to reactive oxygen species generation in cells (Drozdz et al., 1998; Del Bello et al., 1999; Paolicchi et al., 2002).

γGT AND CANCER

Gamma-glutamyl transferases expression has been shown to be increased in numerous cancers. Increased levels have been observed in cancer of the ovary, liver, lung, and breast, and in melanoma and leukemia (Fujisawa et al., 1976; Gerber and Thung, 1980; Dempo et al., 1981; Bard et al., 1986; Corti et al., 2010; Mareš et al., 2012). In many cases the γGT levels are higher in the corresponding primary tumor (Maellaro et al., 2000). An extensive study by Hanigan et al. (1999a) scored the expression of γGT in a variety of tumors. Carcinomas in particular express γGT, with carcinomas of the kidney, liver, and prostate showing strong expression (Hanigan et al., 1999a). Furthermore, some carcinomas of the breast, ovary, uterus, and pancreas were shown to express γGT. On the contrary, non-epithelial malignancies and sarcomas rarely expressed γGT.

Gamma-glutamyl transferases has been considered an early marker of neoplastic transformation. Many early studies have demonstrated in *in vivo* models the appearance of γGT expression in areas previously negative following exposure to carcinogens

(Pompella et al., 2006). The underlying mechanism of this phenomenon remains unclear. A genome-wide analysis of pancreatic cancer implicated GGT1 as playing a role in carcinogenesis (Diergaarde et al., 2010). The proto-oncogene KRAS was shown to be involved in the upregulation of γGT expression. Recently, Moon et al. (2012) have demonstrated that KRAS transformed prostate epithelial cells are more resistant to hydrogen peroxide induced free-radicals than non-transformed cells. They observed an upregulation of GGT2 in the KRAS transfected cells and confirmed its role in resistance to hydrogen peroxide treatment. This has also been observed in colon carcinoma cells where radiation induced γGT activity was mediated through Ras pathway (Pankiv et al., 2006).

The early appearance of γGT in neoplasms suggests the potential for γGT to play a role in tumor progression. γGT has been shown to give cells a growth advantage *in vitro* and *in vivo*. High expression of γGT provides cells with greater quantities of cysteine through the breakdown of extracellular GSH (Gerber and Thung, 1980; Hanigan, 1995; Hochwald et al., 1996). This explains the difference in growth rates of cells when moved from the *in vitro* setting to mice, where extracellular GSH and cysteine is limited *in vivo*. In clones of melanoma cells, the extent of γGT expression was shown to be proportional to the invasive ability of the clone (Supino et al., 1992).

γGT AND DRUG RESISTANCE

Beyond its differential expression in cancer, γGT is considered to be part of a resistance phenotype (Pompella et al., 2006). The main reason for this is the role that γGT plays in maintaining GSH levels within the cell. GSH plays an important part in the detoxification of xenobiotics by binding to a range of agents and this allows for GSH mediated expulsion of these compounds from the cell.

The relationship between γGT and chemotherapy resistance is demonstrated by a number of experiments showing that transfection with γGT both *in vitro* and *in vivo* leads to resistance to members of the platinum drug family, and in particular to *cisplatin* (Hanigan et al., 1999b; Daubeuf et al., 2002; Franzini et al., 2006). Further evidence of the relationship comes from biopsies from ovarian adenocarcinoma patients before and after treatment (cisplatin, chlorambucil, and 5FU). Cells grown from biopsies taken before and after treatment, showed a 6.5-fold increase in γGT activity following treatment (Lewis et al., 1988). However, Schäfer et al. (2001) found no direct link between γGT and resistance, despite an evident growth advantage for γGT overexpressing cells.

Another proposed mechanism for platinum drug resistance is the formation of adducts between the platinum drug and the cysteinyl-glycine product of γGT. These complexes have poor ability to cross the cell membrane; as a result the platinum drug rarely reaches its target (DNA). These adducts have been described in the extracellular media of γGT overexpressing cells and the plasma of patients treated with oxaliplatin (Daubeuf et al., 2002, 2003; Paolicchi et al., 2003; Jerremalm et al., 2006; Corti et al., 2010).

Gamma-glutamyl transferases has also been implicated in resistance to radiation therapy. In lymphoid cells, γGT plays a role in maintaining the intracellular GSH levels that are essential for protection against radiation (Jensen and Meister, 1983). Inhibition of γGT in melanoma cells significantly increased the radiosensitivity of a high γGT variant (Prezioso et al., 1994b). In CC531, a colon cancer cell line, γGT was upregulated in a time and dose-dependent manner to irradiation. This increase in γGT activity was attributed to *de novo* synthesis of the mRNA. It was further demonstrated that signaling through the Ras pathway was responsible for this increase (Pankiv et al., 2006).

HIGH γGT EXPRESSION AND INTRATUMOR DRUG ACTIVATION

The high expression of γGT within aggressive and drug-resistant tumors implies that GSH-conjugated prodrugs, activated at the cell surface by γGT, should be particularly effective against γGT-positive tumors. The metabolism of these compounds by γGT positive tumor cells should produce high local concentrations of bioactive and membrane permeable metabolites that would then block tumor cell proliferation and eventually tumor growth. Increasing the concentration of active metabolites at the tumor site, will subsequently limit the concentration of active drug at other sites, reducing side effects.

Arsenic-based GSH-conjugates

GSAO [4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid] and Darinaparsin (ZIO-101; *S*-dimethylarsino-GSH) are arsenic-based GSH-conjugates with demonstrated antitumor activity evaluated in clinical trials (Don et al., 2003; Dilda et al., 2005b; Diaz et al., 2008; Tsimberidou et al., 2009; Hosein et al., 2012; Horsley et al., 2013). For both drugs, extracellular γGT activity is an essential and limiting step in their activation into membrane permeable compounds (Dilda et al., 2008; Garnier et al., 2014). Indeed, it has recently been demonstrated that tumor γGT could be used for therapeutic delivery (Ramsay et al., 2014).

GSAO. 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid is a prospective cancer drug and has just completed a phase I dose escalation study in patients with solid tumors refractory to standard therapy (Horsley et al., 2013). Treatment was very well tolerated. Of 34 patients, 20 were evaluated for response (receiving two or more cycles of GSAO). Whilst no patient exhibited an objective response, eight had stable disease, with one patient having stable disease for 18 weeks.

4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid consists of a phenylarsenoxide moiety attached by an *N*-acetyl linker to the cysteine thiol of reduced GSH (**Figure 3**). The phenylarsenoxide group is the active moiety, imparting to GSAO its activity by crosslinking closely spaced protein thiols and forming a high affinity ring structure between its arsenic and the thiols (Donoghue et al., 2000). GSAO specifically targets proliferative endothelial cells, which consequently starves the tumor of the nutrients required to support its expanding growth (Don et al., 2003; Dilda et al., 2005b). The GSH moiety contributes to the transport of GSAO in and out of the cell (Dilda et al., 2008, 2005b).

4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid in its original form is essentially membrane impermeable. Upon reaching the cell surface the γ-glutamyl group is cleaved by γGT (Dilda et al., 2008), generating the dipeptide form, 4-(*N*-(*S*-cysteinylglycylacetyl) amino) phenylarsonous acid

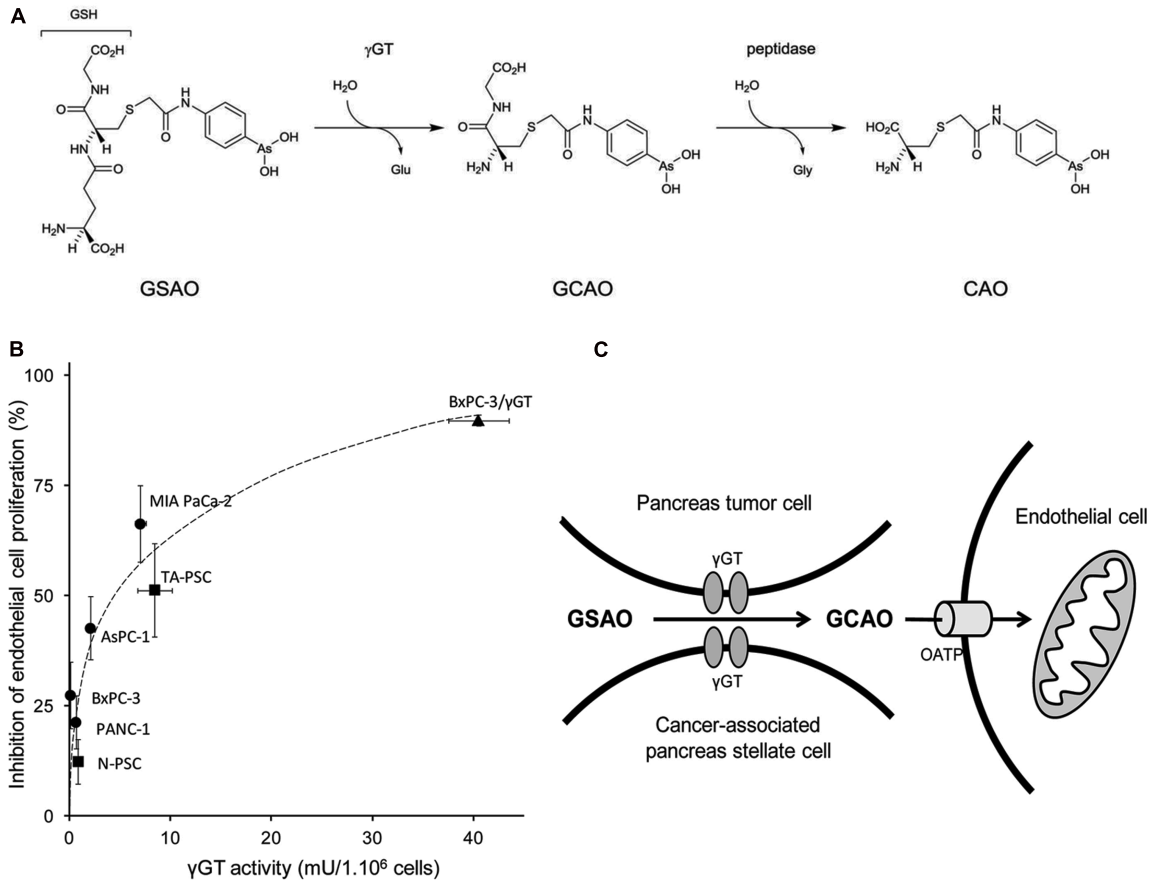

FIGURE 3 | Activation process of GSAO and subsequent activity on endothelial cell proliferation. (A) GSAO activation by γGT and peptidase. **(B)** Pancreatic tumor cell γGT activity positively correlates with GSAO-mediated proliferation arrest of endothelial cells in a transwell model. Pancreatic adenocarcinoma cells are represented by circles and normal and tumor-activated pancreatic stellate cells by squares. BxPC-3 pancreatic tumor cells transfected with γGT are represented as triangles. **(C)** Model of GSAO activation by tumor γGT (from Ramsay et al., 2014).

(GCAO). GCAO is then able to enter the cell through organic anion transporters (OATPs). Within the cell further processing by dipeptidases likely occurs, resulting in the formation of the single amino acid form of GSAO, 4-(*N*-(*S*-cysteinylacetyl)amino)phenylarsonous acid (CAO; Dilda et al., 2008; **Figure 3A**).

The phenylarsenoxide moiety of CAO has been confirmed to crosslink the cysteine residues 57 and 257 of the adenosine nucleotide translocator (ANT; Park et al., 2012). ANT is the most abundant protein found on the inner mitochondrial membrane and it is responsible for the exchange of matrix ATP for cytosolic ADP across the inner mitochondrial membrane. Disruption of its function has been shown to have major impacts on mitochondrial integrity and cell survival (Halestrap et al., 2002; McStay et al., 2002; Don et al., 2003). The calcium-dependent binding of CAO to ANT induces a conformational change (Halestrap et al., 2002) which results in the opening of the mitochondrial permeability transition pore (MPTP) and allows the equilibration of small solutes and the release of pro-apoptotic proteins from the intermembrane space. The equilibration that results leads to a collapse of the proton-motive force across the membrane and a colloid osmotic pressure that causes massive swelling of the mitochondria (McStay et al., 2002).

There are a number of factors that influence GSAO activity: the level of expression of the enzyme responsible for its activation (γGT); intracellular GSH levels; and the expression of the multidrug resistance association proteins, ABCC1 (MRP1) and ABCC2 (MRP2). The combination of low intracellular GSH levels and low expression of ABCC1 and ABCC2 in proliferative endothelial cells accounts for GSAO selectivity and subsequently for GSAO anti-angiogenic properties (Dilda et al., 2005a,b; Park and Dilda, 2010).

Pancreatic tumors display the most prominent stromal/desmoplastic reaction of all epithelial tumors (Apte and Wilson, 2012). Knowing that pancreatic tumorigenesis has been associated with expression of γGT by both cancer and tumor-associated stellate cells (Ramsay et al., 2014), Ramsay et al. (2014) have explored the utility of this enzyme in delivering GSAO to pancreatic ductal adenocarcinoma. They demonstrated that human pancreatic tumor and stellate cells activate/process GSAO into its active metabolite and that γGT activity positively correlates with GSAO-mediated proliferation arrest of co-cultured endothelial

cells (**Figure 3B**). Importantly, tumor γGT activity positively correlates with GSAO-mediated inhibition of pancreatic tumor angiogenesis and tumor growth in mice.

4-(*N*-(*S*-penicillaminylacetyl)-amino)phenylarsonous acid (PENAO), a cysteine mimetic analog of the cysteine-*S*-conjugate metabolite of GSAO has been investigated. In this compound, penicillamine replaces the cysteine moiety of CAO. PENAO was shown to have the same molecular target as GSAO (Dilda et al., 2009; Park et al., 2012). By bypassing the cell surface processing of GSAO, PENAO accumulated 85 times faster in cells (Dilda et al., 2009). This corresponds to an increase in anti-proliferative capacity on a variety of endothelial and cancer cell lines. Interestingly, PENAO has a strong anti-proliferative activity against glioblastoma cell lines (Chung et al., 2011) and primary isolates of diffuse intrinsic pontine glioma (Tsoli et al., 2013). *In vivo*, PENAO demonstrated preclinical activity without signs of toxicity in tumor models of glioblastoma (Chung et al., 2013) and pancreatic carcinoma (Dilda et al., 2009). In the later model, PENAO was approximately 20-fold more efficacious than GSAO (Dilda et al., 2009). Large scale animal toxicity studies demonstrated that PENAO was as well tolerated as GSAO, suggesting an interesting therapeutic window for this compound. PENAO is currently being tested in a clinical Phase I trial in patient with solid tumors refractory to standard chemotherapy. PENAO is another example of a mercapturic acid pathway metabolite with antitumor properties. However, the lack of processing by γGT removes the targeting advantage presented by GSAO.

Darinaparsin. *S*-dimethylarsino-GSH (Darinaparsin; ZIO-101) is an organic arsenical compound currently in clinical development. It has been tested in Human with hematologic malignancies (Hosein et al., 2012; Nielsen et al., 2013) as well as solid tumors (Tsimberidou et al., 2009; Wu et al., 2010). Significantly more potent and better tolerated than arsenic trioxide, Darinaparsin showed encouraging responses in T-cell lymphoma (Hosein et al., 2012) and AML (Nielsen et al., 2013) patients. However, in a phase II evaluation of the compound in hepatocellular carcinoma, no objective response was shown (Wu et al., 2010).

Darinaparsin belongs to the class of organic arsenical compounds that are generally considered less toxic than inorganic ones (Waxman and Anderson, 2001; Dilda and Hogg, 2007). It was synthesized by conjugating GSH to dimethyl arsenic (**Figure 4**). Darinaparsin shares some characteristics with other arsenicals but has also unique properties. As do other arsenicals, it induces G_2/M cell cycle arrest and triggers apoptosis through disruption of mitochondrial functions and JNK activation and is responsible for reactive oxygen species production (Diaz et al., 2008). However, unlike arsenic trioxide, GSAO (Dilda et al., 2005b) or PENAO (Dilda et al., 2009), Darinaparsin activity is unaffected by the expression of ABCC1 (MRP-1), modulation of GSH levels (Diaz et al., 2008) and heme-oxygenase inhibition (Garnier et al., 2013).

Darinaparsin was characterized *in vitro* as a potent inducer of growth arrest and apoptosis in a range of hematologic malignancies such as acute promyelocytic leukemia (APL), acute lymphoblastic leukemia, B cell lymphoma, and multiple myeloma (Diaz et al., 2008; Matulis et al., 2009). In contrast to arsenic trioxide (Lallemand-Breitenbach et al., 2012) and like PENAO and melarsoprol (Chen et al., 2003), Darinaparsin, at concentrations that induce apoptosis, does not induce PML/RAR degradation in APL cells. In the context of solid cancer, Darinaparsin was found to have preferential cytotoxic and radiosensitizing effects as compared with normal cells both *in vitro* and in a clinically relevant model (Tian et al., 2012, 2013).

It was recently demonstrated that Darinaparsin, which is essentially a GSH *S*-conjugate of dimethylarsenic, needs to be processed at the cell surface before exerting its activity on various cancer cells. The GSH moiety of the drug has to be processed by γGT before further processing by dipeptidases to generate dimethylarsinocysteine (DMAC), a cysteine *S*-conjugate which is imported via cystine transporters (Garnier et al., 2014; **Figure 4**). Whether DMAC could be subsequently transformed intracellularly into (i) a highly reactive and cytotoxic thiol through β-lyase activity or (ii) dimethylarsenite/dimethylarsenate is not known. Similarly to what was observed in the case of GSAO and CAO (Dilda et al., 2008), Garnier et al. (2014) demonstrated that DMAC recapitulates the effects of Darinaparsin and that γGT is the first and limiting step in the activation of the drug. They also linked Darinaparsin efficacy to the level of expression of cystine/cysteine importing systems in cancer cells.

S-nitrosoglutathione

S-nitrosoglutathione (GSNO) is the *S*-nitrosated derivative of GSH and is thought to be a critical mediator of the downstream signaling effects of NO (Broniowska et al., 2013). GSNO plays roles as a carrier for NO and in protein *S*-nitrosation or *S*-glutathiolation (Broniowska et al., 2013). GSNO can be cleaved by γGT (Hogg et al., 1997; Angeli et al., 2009; Bramanti et al., 2009), generating *S*-nitrosocysteinylglycine and releasing NO *in vitro*. GSNO has been tested in a number of clinical trials, the majority focusing on the cardiovascular effects of GSNO as a NO donor (Broniowska et al., 2013). As do other NO-donating compounds, GSNO induces oxidative stress and initiates apoptosis (Turchi, 2006). GSNO promotes NO-induced apoptosis in colon carcinoma cell lines through depletion of intracellular GSH and the release of NO, (Ho et al., 1999; Liu et al., 2003). In a variety of cell lines, GSNO treatment combined with a MEK inhibitor was shown to inhibit the proliferation and invasive phenotype of the cells (Furuhashi et al., 2012). In T and B lymphocytes the anti-proliferative effect of GSNO was dependent on γGT activity (Henson et al., 1999). The anti-proliferative activity of GSNO, combined with the overexpression of γGT in tumor cells, suggests the possibility of its utility in cancer therapy. This requires further understanding of the roles that NO plays in cancer.

In the light of the promising results obtained, notably with the arsenical-based GSH-conjugates, it appears that tumor γGT could potentially be employed in drug targeting and delivery. In addition to cleavage of the γ-glutamyl from a GSH moiety, γGT can also catalyze the cleavage of γ-glutamyl moieties from a variety of compounds. This property along with tissue/tumor expression of γGT was used to develop γ-glutamyl prodrugs.

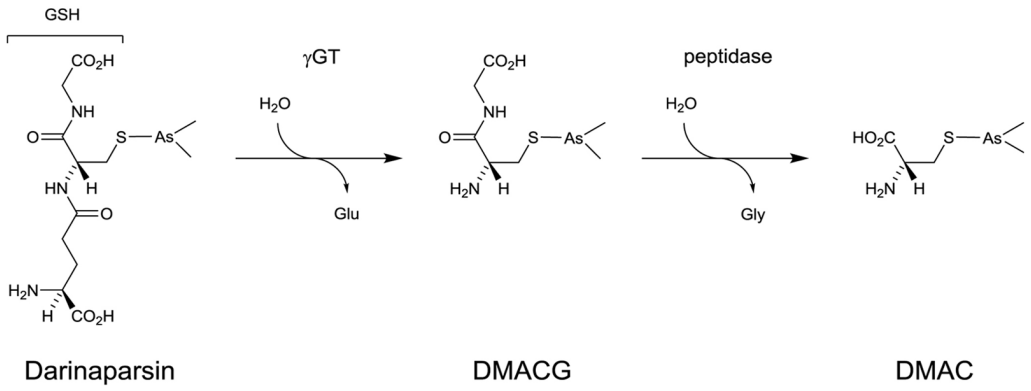

FIGURE 4 | Activation process of Darinaparsin.

γ-glutamyl conjugates and N acetyl γ-glutamyl conjugates

Due to the tissue distribution of γGT, γ-glutamyl prodrugs have been mostly developed for kidney applications. An anti-nociceptive prodrug, γ-glutamyl-dermorphin, was explored by comparing the pain threshold of mice (Misicka et al., 1996). More extensively investigated was the γ-glutamyl conjugate of L-DOPA (dihydroxyphenylalanine). This prodrug provides the precursor of dopamine, DOPA, upon activation and was explored as a potential renal vasodilator (Wilk et al., 1978; Worth et al., 1985; Sadiq et al., 2000). Clinical testing of the prodrug determined that despite reasonable kidney specificity it had low bioavailability (Lee, 1990). A number of other compounds were conjugated to a glutamyl group and tested; however none were approved for use (Huttunen and Rautio, 2011).

In terms of potential antitumor activity, γ-glutamyl-protected *N*-hydroxyguanidines (NHGs) have been developed to explore the ability to deliver NO to the kidney (**Figure 5A**). The NHGs include N^w-hydroxy-L-arginine, which is an intermediate in the NO synthase synthesis of NO (Zhang et al., 2013b). Whilst promising results were seen, there was a propensity for the potential compounds to cyclize (Zhang et al., 2013a,b) suggesting that NHG conjugation to GSH may be more stable. (Zhang et al., 2013a). As described above for PABA/NO, JS-K, and GSNO, the NHGs, in accordance with γGT expression patterns, contribute to chemotherapy by producing locally high levels of NO which can rapidly react with $O_2^{\cdot}-$ to generate the potent oxidant peroxynitrite that, in turn causes extensive cellular damage, including the nitration of protein tyrosine residues (Kirsch et al., 2001).

Glutamylated phenolic amine compounds have been shown to be activated by γGT (**Figure 5B**). γ-L-glutaminyl-4-hydroxybenzene and γ-L-glutaminyl-4-iodobenzene have been demonstrated to be activated by tyrosinase (Prezioso et al., 1993) and γGT (Prezioso et al., 1994a). Both compounds were shown to have greater cytotoxicity in melanoma cell lines when γGT activity was not inhibited (Prezioso et al., 1994a).

γGT-activated prodrugs and potential nephrotoxicity

Gamma-glutamyl transferase being the initial and limiting step in γGT-activated prodrugs processing and because γGT is highly expressed on the luminal surface of the proximal tubules in the kidney, potential nephrotoxicity could have been a limitation in the usage of this class of compound when administered systematically. In the case of GSAO, a reversible renal toxicity described in preclinical studies at the maximal tolerated dose was not observed in human during clinical Phase I (Horsley et al., 2013). Similarly, Darinaparsin, when employed in patients (Tsimberidou et al., 2009; Wu et al., 2010; Hosein et al., 2012) didn't display any toxicity profile related to renal dysfunction at dose-limiting toxicity (DLT). Taken together, these clinical studies indicate that the γGT-activated prodrugs have not necessarily nephrotoxic properties. This type of adverse effects is most likely dependent on the nature of the metabolites produced by the mercapturic acid pathway and particularly if the end products are substrates for cysteine conjugate β-lyases. This enzyme is notably responsible for the nephrotoxicity of cisplatin (see below). However, recent studies demonstrated that the modulation of renal GSH content could prevent such toxicity (Firdous and Kuttan, 2012; Sener et al., 2012; Ghosh et al., 2013).

β-LYASE AND GENERATION OF CYTOTOXIC PRODUCTS

Cysteine conjugates produced by dipeptidases or amino peptidases following the GST and γGT steps, are transformed by cysteine conjugate β-lyases present in the cytosol and mitochondria. The β-lyases catalyze the β-elimination of various L-cysteine *S*-conjugates to the corresponding thiols (Cooper, 1998). The thiols released can be either toxic or pharmacologically active for the cells responsible for their production (Commandeur et al., 1995). Due to the high expression of cysteine conjugate β-lyase in the proximal tubule of the kidney, administration of cysteine-*S*-conjugates of haloalkenes to rodents results in selective nephrotoxicity (Stevens et al., 1989; Hayden and Stevens, 1990).

The specific localization along with intense expression of these enzymes in renal carcinoma has led to the design of kidney-selective prodrugs that bypass earlier steps of the mercapturic acid pathway (GST, γGT, and peptidase): these compounds include *S*-(guanin-6-yl)-L-cysteine (Elfarra et al., 1995), *S*-(6-Purinyl)-L-cysteine (Hwang and Elfarra, 1991; Lash et al., 1997) and selenocysteine-conjugates (Commandeur et al., 2000; **Figure 5C**). The potential of these types of compounds as kidney-selective antitumor prodrugs has been demonstrated in tissue distribution

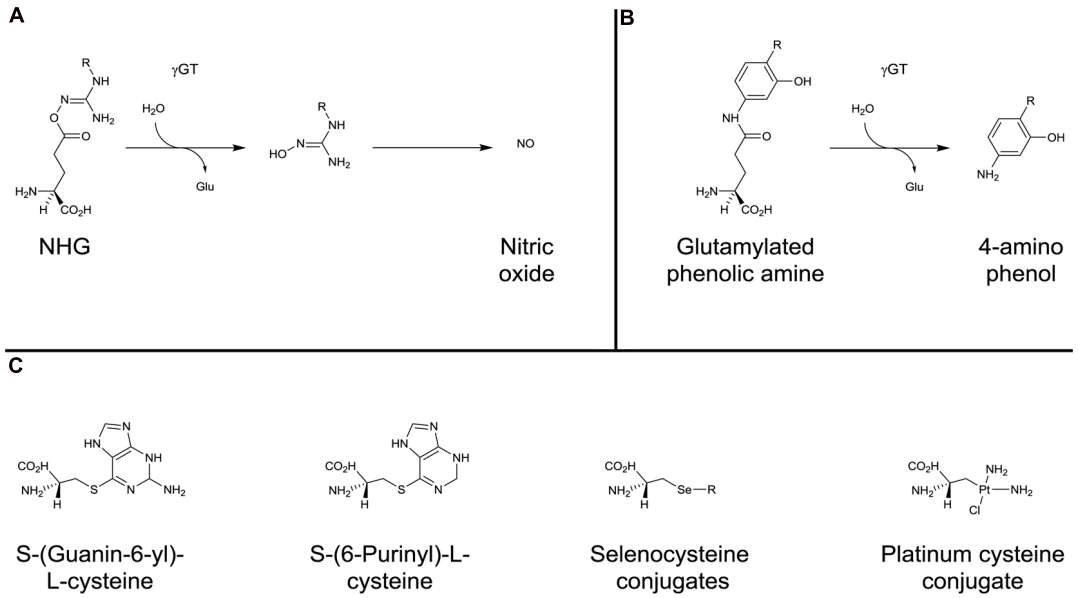

FIGURE 5 | γ-glutamyl conjugates and β-lyase substrates. Activation of *N*-hydroxyguanidines **(A)** and glutamylated phenolic amines **(B)** by γGT. **(C)** examples of cysteine- and selenocysteine-conjugates activated by β-elimination catalyzed by β-lyase.

experiments. However, because of a lower ratio of kidney/liver β-lyase activity in human than rodents, kidney selectivity remains to be confirmed.

Cisplatin is one of the most effective anticancer agents in the treatment of solid tumors, including, breast, testicular, and ovarian cancer. The drug binds DNA, which is toxic to dividing tumor cells. However, therapy with cisplatin is notably limited by a toxicity mechanism in the non-dividing proximal tubule cells of the kidney that is distinct from DNA cross-linking. It was demonstrated that cysteine *S*-conjugate β-lyase highly expressed in the kidney was responsible for the observed nephrotoxicity (Zhang and Hanigan, 2003; Townsend et al., 2003). Even if cysteine *S*-conjugate β-lyase is responsible for the synthesis of the highly reactive and cytotoxic thiol version of cisplatin (**Figure 1**), the activation of cisplatin into a nephrotoxin relies on the three previous steps of the classical mercapturic acid pathway involving GSTs (GSH conjugation), γGT (production of CisPt-cys-glu) and dipeptidase (production of CisPt-cys; **Figure 5C**). Indeed, the inhibition of γGT or cysteine *S*-conjugate β-lyase (both highly expressed in proximal tubule cells of the kidney) blocks the nephrotoxicity of cisplatin in mice (Townsend et al., 2003). In this process, the action of γGT is a key and limiting step as it transformed the essentially membrane impermeable CisPt-GSH into a membrane permeable CisPt-cys-glu that can be taken up by the cells.

CONCLUSION

The traditional approach to identify potential cancer therapies has been to identify a drug that helps the greatest number of patients. In doing this, the potential of therapies that have a significant impact on a small subset is lost within the larger sample population. The current trend to target specific abnormalities in cancer allows for the development of predictive markers and companion tests to identify which patients have a particular tumor characteristic. From this stems the ability to identify the patient population that will (or will not) respond to the drug. Developing new therapies with the target population in mind will facilitate the development of drugs that will induce a greater tumor response for individual patients. For this to occur, a marker that predicts patient response to the drug is essential.

This review recapitulates the different drug strategies involving GSH-conjugates, and the enzymes of the mercapturic acid pathway, employed over the last twenty years. Amongst the long list of compounds investigated, a limited number of GSH-conjugates have recently progressed towards clinical trials for the treatment of cancer patients. Both GST-activated nitrogen mustard (TLK286) and γGT-activated arsenic-based (GSAO and Darinaparsin) prodrugs have displayed promising activities along with safe profiles.

Given the complexity of mechanisms involved in drug resistance and survival pathways of tumor cells, combination strategies of GSH-conjugates with conventional chemotherapy or targeted drugs should provide very interesting perspectives for the treatment of drug resistant tumors. This approach has already been explored successfully with TLK286 when combined with pegylated liposomal doxorubicin (Kavanagh et al., 2010; Vergote et al., 2010) or with carboplatin plus paclitaxel (Sequist et al., 2009). Moreover, knowing that the combinations of arsenic trioxide with targeted drugs such as mTOR (Guilbert et al., 2013; Iwanami et al., 2013), EGFR (Noh et al., 2010; Kryeziu et al., 2013), or proteasome inhibitors (Jung et al., 2012) demonstrate potent profiles on a variety of malignancies, it would be of great interest to investigate the antitumor properties of such inhibitors in combination with the arsenical-based GSH-conjugates GSAO and Darinaparsin.

Increased GST and/or γGT tumor expression, generally considered as a bad prognosis marker (associated with drug resistance and disease progression), and could potentially be used as a predictive marker, allowing doctors, after biopsy, to identify which patients will respond best to GST or γGT-activated prodrugs. Predictive biomarkers provide a number of benefits, including: reduced unnecessary treatment; reduced quantity of adverse events; reduced drug attrition rates; improved therapeutic benefit; and better control of medical costs (La Thangue and Kerr, 2011). Whilst taking the time to test for GST and/or γGT tumor expression may slightly delay treatment, it will ensure that GSH-conjugates anticancer prodrugs will be administered to the patients that are more likely to respond.

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Glutathione levels modulation as a strategy in host-parasite interactions—insights for biology of cancer

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Parasitic organisms establish a symbiotic association with individuals of a different species—the host—to obtain the metabolic resources needed for their survival and reproduction. These antagonistic associations are characterized by a complex "host-parasite molecular cross-talk," shaped by evolutionary processes largely driven by the requirement of making host's tissues more accessible to a successful colonization and exploitation (Hébert and Aubin-Horth, 2014). In a large number of cases, parasitism not only impairs host physiology and reproduction, but may even culminate with its death or complete consumption. This is the rule in insects, which include the largest number of species with parasitic life habits, characterized by subtle virulence strategies, often shared among organisms belonging to distant phylogenetic groups (Pennacchio and Strand, 2006). The astonishing diversity and the unexpected similarities shared with unrelated taxonomic entities offer the opportunity of insightful comparisons among virulence strategies targeting conserved molecular pathways, as a result of convergent evolutionary patterns.

Here we focus on a comparative analysis of a peculiar strategy of host physiological regulation, targeting the redox homeostasis, which appears to modulate parasitic interactions both at the organism and cellular level.

Insect studies, dealing with parasitism of aphids, have shown that the disruption of host GSH pool and metabolisms significantly contributes to its physiological regulation and castration (Pennacchio and Mancini, 2012). The parasitic wasp *Aphidius ervi* injects at the oviposition into host aphids a venom containing large amounts of a gamma-glutamyltransferase (*Ae-GGT*), which triggers apoptosis in the upper part of the aphid ovarioles and therefore blocks oogenesis (Falabella et al., 2007). How this selective targeting is achieved and modulated at molecular level is still obscure; however, preliminary data indicate that in parasitized host aphid a depletion of GSH occurs, which primarily involves ovarian tissue (Masi and Pennacchio, unpublished results). It does not require a leap of imagination to speculate that injected *Ae*-GGT can compete with endogenous GGT for substrate GSH, thus interfering with the regular GSH cycle and exposing GSH-depleted cells to an oxidative stress, which ultimately triggers apoptosis. However, it is also reasonable to consider that the alteration of cell survival/apoptosis balance might ensue from the known pro-oxidant effects of GGT activity, due to increased metal-reducing ability of the GSH metabolite cysteinylglycine and extracellular production of reactive oxygen species (Paolicchi et al., 2002). The presence of a GGT in the genome of an entomopoxvirus associated with the parasitic wasp *Diachasmimorpha longicaudata* (Hashimoto and Lawrence, 2005), injected at the oviposition and used as a delivery system of virulence factors, indicates that this enzyme may have additional regulatory roles of host physiology, which remain largely elusive. The possible negative impact on host immunity (Clark et al., 2010) is certainly worth of consideration.

Studies on virulence factors of microrganisms have documented that the invasion strategies of selected pathogenic bacteria also target host GSH metabolism. Indeed, it has been shown that GGT activity of *Helicobacter pylori* and *H. suis*, the agents responsible of peptic ulcer, can exert antiproliferative and pro-apoptotic effects in gastric epithelial cells (Schmees et al., 2007; Gong et al., 2010; Ricci et al., 2014). These effects are triggered by GGT-dependent metabolism of GSH and production of H_2O_2, which result in the activation of NF-kB, up-regulation of interleukin-8 and increased oxidative DNA damage (Gong et al., 2010; Ricci et al., 2014). Paradoxically enough, supplementation with glutathione of *H. pylori* GGT-treated cells strongly enhanced the harmful effects (Flahou et al., 2011; Zhang et al., 2013). By confocal microscopy, *H. suis* outer membrane vescicles (OMV)—submicroscopic structures 20–50 nm in diamater, budding from the cell surface—were identified as carriers of *H. suis* GGT, capable of delivering the enzyme to the deeper mucosal layers (Zhang et al., 2013). In association with such membranous structures, active GGT from *H. suis* in fact translocates across the epithelial layers and can access lymphocytes residing in the lamina propria of gastric mucosa. The result of this intriguing process appears to be an inhibition of lymphocyte proliferation, i.e., a perturbation of host immunity and a facilitation of bacterial infection (Oertli et al., 2013). Future studies will likely expand the number of examples of GSH-based host-parasite interactions, as possibly in the case of pro-apoptotic activity of GGT released by *Campylobacter jejunii* (Barnes et al., 2007). Cellular GSH appears, thus, to represent a conserved target for parasitic

(micro)organisms which aim at altering host redox homeostasis to weaken its immune defenses, using GGT as a key-element of a virulence strategy.

It is possible to further elaborate on this concept by jumping in the field of cancer biology and taking into account the "parasitic" behavior exhibited by malignant cells spreading across tissues and organs of the patient (the "host"). GGT activity is in fact expressed in a number of malignant tumors, and expression levels often increase along with progression of the disease and appearance of more invasive phenotypes (reviewed in Pompella et al., 2006). Importantly, recent studies showed that active GGT can be released from cells, including cancer cells (Franzini et al., 2009), in association with submicroscopic vesicles, 20–40 nm in diameter, resembling exosomes (Fornaciari et al., 2014). The similarity of such structures with GGT-rich OMV particles of *H. pylori* and *H. suis* is indeed obvious. GGT activity of cancer cells can affect intracellular redox equilibria (Pompella et al., 2007), and produces in addition significant extracellular effects, on the S-thiolation status of extracellular proteins (Corti et al., 2005), as well as on the redox status and ligand binding affinity of cell surface receptors related with cell survival/apoptosis balance (Dominici et al., 2004). The question therefore arises, whether GGT-rich exosomes shed by cancer cells can produce in host's surrounding tissues effects comparable to those reported for *Ae*-GGT or *Helicobacter* GGT, possibly resulting in facilitation of malignant cells survival and diffusion.

Collectively, these GSH/GGT-dependent processes described in evolutionary distant organisms and different cell populations further corroborate the pivotal importance of redox homeostasis in the modulation of health and disease conditions; shedding light on regulatory elements of these convergent parasitic strategies will likely allow the identification of new potential targets for therapy.

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