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Emerging Mechanisms of Glutathione-dependent Chemistry in Biology and Disease†

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Abstract

Glutathione has traditionally been considered as an antioxidant that protects cells against oxidative stress. Hence, the loss of reduced glutathione and formation of glutathione disulfide is considered a classical parameter of oxidative stress that is increased in diseases. Recent studies have emerged that demonstrate that glutathione plays a more direct role in biological and pathophysiological processes through covalent modification to reactive cysteines within proteins, a process known as S-glutathionylation. The formation of an S-glutathionylated moiety within the protein can lead to structural and functional modifications. Activation, inactivation, loss of function, and gain of function have all been attributed to S-glutathionylation. In pathophysiological settings, S-glutathionylation is tightly regulated. This perspective offers a concise overview of the emerging field of protein thiol redox modifications. We will also cover newly developed methodology to detect S-glutathionylation *in situ*, which will enable further discovery into the role of S-glutathionylation in biology and disease.

Keywords

Glutaredoxin-1; Protein S-glutathionylation; Redox; Biotin Switch

PROTEIN CYSTEINES AS REDOX SENSORS

Oxidants have traditionally been considered as damaging molecules that adversely affect all macromolecules, thereby contributing to an array of diseases ranging from chronic inflammatory disorders, degenerative processes to cancer [Anathy et al., 2012a; Xiong et al., 2011]. Along with the discovery of non-phagocytic oxidases which are expressed in virtually every cell type [Altenhofer et al., 2012], it has become apparent that oxidants have important physiological functions in biological processes, such as embryogenesis, control of circadian rhythms, and neutrophil recruitment [Edgar et al., 2012; Kuzin et al., 1996; Marcos et al., 2010], among others. This biological function of oxidants is believed to be

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largely attributed to oxidation of protein cysteines, which can be considered redox sensors that transduce an oxidative signal into a biological response [Anathy et al., 2012a]. Protein cysteines are evolutionarily highly conserved and sparingly used throughout the proteome, suggesting tight control of cysteine-dependent biological functions. Protein cysteines have a wide-ranging reactivity towards oxidants, which depends on their pKa [Dalle-Donne et al., 2007; Rutkevich and Williams, 2012] and the fraction of cysteine that is present in the deprotonated, thiolate state (S^-) which is the main target for oxidation. The pKa of a cysteine is controlled by the charge of flanking amino acids, within the three dimensional conformation or quaternary state [Marino and Gladyshev, 2012]. Therefore, although a protein can have multiple cysteines, only a few may be targets for oxidation.

A reactive cysteine in the thiolate state can be oxidized in diverse manners (Fig. 1). In response to hydrogen peroxide (H_2O_2), or related oxidants, a sulfenic acid intermediate (hydroxylated state, SOH) is formed. The SOH intermediate form is thought to be the initial oxidation step (“gateway oxidation”) that can give rise to other products. The sulfenic acid intermediate can be stabilized, or give rise to sulfenamides (SN), or disulfide bonds (S-S) which can occur within a protein (intramolecular disulfide) or between proteins (intermolecular disulfides) [Anathy et al., 2012b; Klomsiri et al., 2011]. Sulfenic acids can also be further oxidized to sulfinic (SO_2H) and sulfonic (SO_3H) intermediates [Dalle-Donne et al., 2007; Winterbourn and Hampton, 2008], and sulfenamides can be further oxidized to sulfinamides (SON) or sulfonamides (SO_2N) [Klomsiri et al., 2011]. Reactive cysteines are also subject to S-nitrosylation (also known as S-nitrosation (SNO)), mediated by nitric oxide, S-nitrosothiols, or more highly reactive nitrogen species such as nitrogen dioxide, or peroxynitrite [Hess and Stamler, 2012]. Sulfhydration of protein cysteines (the formation of a persulfide, SSH bond), is catalyzed by the gaseous molecule hydrogen sulfite (H_2S), which, like nitric oxide, is believed to play important physiological functions [Paul and Snyder, 2012]. The thiol antioxidant, glutathione, can also become covalently attached to protein cysteines, a modification known as S-glutathionylation, S-glutathiolation, or protein mixed disulfides (PSSG), as will be further discussed below [Anathy et al., 2012b; Xiong et al., 2011]. Similarly, cysteinylation represents the conjugation of cysteine to a reactive protein cysteine, (PSSC) [Coppo and Ghezzi, 2011]. Cysteines also are targeted by a number of reactive molecules including, lipid peroxidation products (including hydroxynonenal), cyclopentanone prostaglandins, alkylating species (acolein), and avicins, which can be formed endogenously, or encountered through environmental insults [Finkelstein et al., 2005; Rossi et al., 2000], and by acylation, the latter having important roles in localizing proteins to the membranes [Resh, 2006]. This complicated repertoire of possible cysteine oxidations illustrates the complexity as well as versatility of cysteine-dependent modifications, and have led to coining cysteine as a “chameleon” amongst amino acids [Jacob, 2011].

It is currently believed that the type of cysteine oxidation controls the biological response, as well as the fate of the oxidized protein [Finkel, 2011]. Reversible cysteine oxidations can be restored to the sulfhydryl group by enzyme systems such as thioredoxins (Trx) and glutaredoxins (Grx). Overoxidized cysteines, such as sulfinic and sulfonic acids are not readily reversed [Finkel, 2011; Klomsiri et al., 2011]. Of interest are recent findings demonstrating that sulfiredoxins (Srx) regenerate peroxiredoxins (Prx) in the sulfinic state, therefore act as sulfinic acid reductases. Overoxidized peroxiredoxins (containing cysteines in the SO_2H and SO_3H states) pack into higher molecular donut shaped structures that no longer have catalytic activity, but instead act as chaperones [Klomsiri et al., 2011]. While some cysteine oxidations involve relatively modest structural and conformational changes, others such as S-glutathionylation (the addition of three amino acids), might constitute a substantial conformational change. The hierarchy of various cysteine oxidations in the control of biological responses remains unknown. It has been speculated that certain

oxidations, notably S-nitrosylation, and sulfhydration occur during normal cellular processes while others, such as S-glutathionylation, may predominantly occur under conditions of cellular stress, although this notion remains an area of active investigation and likely depends on the cell type, biological context, as well as the protein target [Janssen-Heininger et al., 2008]. Certain types of cysteine oxidation may preferentially occur in subcellular compartments where the local redox environment may be facilitating such oxidations. In this regard, the endoplasmic reticulum (ER) is highly oxidizing and provides the essential environment to create disulfide bridges, catalyzed by protein disulfide isomerases (PDI), essential to stabilize proteins destined for the secretory pathway [Bulleid and Ellgaard, 2011; Chakravarthi et al., 2006]. Similarly, the reaction between nitric oxide and oxygen is greatly enhanced in hydrophobic compartments, facilitating S-nitrosylation [Janssen-Heininger et al., 2008]. Increasingly well appreciated is the concept that redox-based regulation of biological processes is highly compartmentalized, and oxidation of selective target proteins is controlled by localized production of oxidants. Examples of compartmentalized target oxidations include the oxidation of protein tyrosine 1B (PTP1B) via NADPH oxidase 4 (Nox4)-catalyzed H_2O_2 production in the ER [Chen et al., 2008], or Duox-dependent oxidation of Src kinases in membrane signaling complexes [Sham et al., 2013; Yoo et al., 2012]. Similarly, NADPH oxidase 2 (Nox2)-linked oxidant production in endosomes was demonstrated to be required for recruitment of IL1R effectors, and for subsequent propagation of IL1R signaling [Oakley et al., 2009b]. Compartmentalized oxidations in membrane bound organelles have been referred to as “redoxosomes” [Oakley et al., 2009a].

Diverse classes of enzymes have emerged that collectively control the thiol redox state or their target proteins, and a number of these play an important function in the ER (Fig. 2). Among these thiol-oxidoreductases are protein disulfide isomerases (PDI) [Bulleid and Ellgaard, 2011], thioredoxins (Trx), sulfiredoxins (Srx), and glutaredoxins (Grx), As was stated above, the family of PDI catalyzes two-electron thiol disulfide exchange reactions that lead to formation of disulfide bridges in the client proteins. Sulfhydryl oxidases (SOX), such as ER oxidoreductin 1 (Ero1), quiescin sulfhydryl oxidase (QSOX), and vitamin K epoxide hydrolase (VKOR) also create disulfide bridges into proteins and have been identified as important catalysts in disulfide bond formation within the ER [Bulleid and Ellgaard, 2011; Rutkevich and Williams, 2012]. Similarly, glutathione peroxidase (GPX) 7 and 8 are ER localized enzymes that function as PDI peroxidases and contribute to disulfide bond formation [Nguyen et al., 2011]. Although peroxiredoxins (Prx) are known to detoxify H_2O_2, oxidized peroxiredoxin 4 (Prx4) was recently demonstrated to play a role in oxidation of PDI members, thereby regulating disulfide formation in the ER [Tavender et al., 2010; Zito et al., 2010]. Trx reduce disulfide bonds in proteins to the reduced sulfhydryl state (SH). Trx is regenerated via thioredoxin reductases (TrxR) at the expense of NADPH, and plays an important role in cellular redox homeostasis. In addition to Trx, TrxR, and NADPH, thioredoxin-interacting protein (TXNIP) is also a component of the Trx system [Mahmood et al., 2013]. More recently, Trx have also been shown to play a role in denitrosylation reactions [Benhar et al., 2008]. Trx have emerged as critical regulators or biological processes and numerous Trx target proteins have now been identified [Lee et al., 2013]. As was stated earlier, Srx are enzymes that regenerate 2-Cys peroxiredoxins in the sulfinic state [Jeong et al., 2012], and it is not clear whether additional Srx targets exist. Relevant to this perspective are findings demonstrating that Srx play a role in de-glutathionylation reactions [Findlay et al., 2006; Jeong et al., 2012].

GLUTATHIONE: THIOL ANTIOXIDANT AND ROLE IN POST-TRANSLATIONAL MODIFICATION

Glutathione (GSH) is a tri-peptide thiol antioxidant molecule that is present within cells at millimolar concentrations (1–10mM). In response to an increased oxidative burden,

glutathione becomes oxidized to its disulfide form (GSSG). The disulfide form, GSSG can be reduced back to GSH via glutathione reductase, at the expense of oxidation of NADPH, which is re-generated via the pentose phosphate pathway. In the cytosol, the ratio of oxidized to reduced GSH is around 100:1. In contrast in the ER, which has a more oxidizing environment to permit oxidative protein folding, the ratio of GSH/GSSG has been predicted to be around 3:1 [Bulleid and Ellgaard, 2011]. The extent of oxidation of GSH is often used as a measure of "oxidative stress", which can be measured in various biological fluids, including plasma, and is known to increase in an array of human diseases. However, an emerging body of literature suggests that GSH constitutes more than a thiol antioxidant, and that glutathione plays a prominent role in redox-dependent biological responses, via the aforementioned S-glutathionylation, i.e., the conjugation of glutathione to reactive cysteines in proteins (PSSG) [Janssen-Heininger et al., 2008; Stroher and Millar, 2012; Xiong et al., 2011]. It was traditionally viewed that conjugation of GSH to oxidized protein cysteines merely serves a biochemical protective mechanism in order to prevent irreversible over-oxidation of protein cysteines, such as sulfonic (SO_3H) containing residues. More recently, however, PSSG has been revealed to have an important role in the regulation of protein structure and function, and to reversibly impact biological signals [Dalle-Donne et al., 2007; Janssen-Heininger et al., 2008; Mieyal et al., 2008]. Of interest are recent findings that glutathione S-transferase pi (GSTP) can act as a catalyst in S-glutathionylation reactions [Xiong et al., 2011]. GSTP catalyzes S-glutathionylation of oxidized peroxiredoxin-6, permitting regeneration of the active enzyme [Manevich and Fisher, 2005]. In response to reactive nitrogen species, GSTP also plays a critical role in formation of PSSG [Townsend et al., 2009].

GLUTAREDOXINS

Mammalian dithiol glutaredoxins (Grx) contains a thioredoxin motif Cys-X-X-Cys within the active site, and can catalyze reversible S-glutathionylation and de-glutathionylation reactions. The family of Grx represents a set of heterogeneous proteins that is conserved across many species. Two major classes of Grx have been indentified to date. The dithiol Grx with an active site consensus motif Cys-Pro-Tyr-Cys, and the monothiol Grx with a Cys-Gly-Phe-Ser consensus site. Monothiol Grx can be additionally distinguished into single domain monothiol Grx and multi-domain monothiol Grx that contain an N-terminal Trx-like domain, in addition to one to three monothiol Grx domains. Four Grx have been isolated in human cells. Grx1 and 2 are dithiol Grx, while Grx 4 and 5 are monothiol Grx. Grx1 (active site; Cys-Pro-Tyr-Cys) is cytosolic, while Grx2 (active site; Cys-Ser-Tyr-Cys) is localized to the mitochondria. Human Grx3 is a multidomain monothiol Grx, with an N-terminal Trx-like domain and two additional monothiol domains (each containing Cys-Gly-Phe-Ser), while Grx5 is a single domain monothiol Grx (Cys-Gly-Phe-Ser) that is expressed in mitochondria [Lillig et al., 2008]. Many Grx can carry out dithiol reactions. Mammalian Grx1 carries out de-glutathionylation reactions via a monothiol reaction mechanism. In this reaction, the N-terminal cysteine of mammalian Grx1 undergoes a thiol disulfide exchange with the S-glutathionylated protein, which results in reduction of the protein thiol, and a glutathionylated intermediate of Grx1 (Grx1-SSG). Grx1-SSG which in turn is reduced by glutathione, giving rise to glutathione disulfide (GSSG), which as mentioned above can be reduced via glutathione reductase [Stroher and Millar, 2012]. Under physiological conditions, where reduced GSH levels are high, the action of Grx1 is to de-glutathionylate target proteins, while under conditions where glutathione is largely oxidized, Grx1 can also catalyze glutathionylation reactions.

Grx are beginning to be implicated in diverse diseases that include allergic airways disease, cardiovascular disease. As an example, Grx1 content was increased in sputum of patients with asthma in comparisons to controls, while PSSG content was decreased. Interestingly, a

significant negative correlation between Grx1 and lung function was observed, and suggested that better lung function is associated with lower Grx1 and higher PSSG in sputum [Kuipers et al., 2013]. In patients with chronic obstructive pulmonary disease, Grx1-expressing macrophages decreased, and significantly correlated with changes in lung function [Peltoniemi et al., 2006], suggesting complex roles of Grx1 in diverse disease settings. In mice with allergic airways disease, a model of asthma, expression of Grx1 but not Grx2 increased in comparison to control groups [Reynaert et al., 2007]. Absence of the glutaredoxin-1 gene (*Glrx1* −/− genotype) led to faster resolution of airways hyperresponsiveness to an inhaled bronchoconstricting agent compared to WT mice, in association with a higher extent of protein S-glutathionylation [Hoffman et al., 2012]. Altogether, these studies in patients and mouse models of disease suggest a contribution of Grx1 in disease pathogenesis. However the critical cell type wherein Grx1 exerts its effects, as well as the target proteins of Grx1 that are controlled through S-glutathionylation remain unknown, and warrant additional investigation into spatial expression patterns of Grx1, and protein-S-glutathionylation, as will be discussed next.

DETECTION OF S-GLUTATHIONYLATION

Classical methods to detect S-glutathionylation involve direct analysis of GSH after chemical reduction of precipitated proteins from plasma or tissue homogenates. After removing the non-protein supernatant, and washing the protein-containing pellet, glutathione is next released (by chemical reduction) from the proteins and detected using various biochemical assays [Rahman et al., 2006]. While such methodology provides quantitative information about the extent of PSSG, typically in the nmol/mg protein range, it provides no insight into the location within a complex tissue in which PSSG is affected. In order to overcome this limitation, our laboratory recently developed methodology that utilizes the properties of Grx1 in order to reveal patterns of PSSG within tissues, and changes that may occur in disease settings. This method can be used in paraffin-embedded tissues, and hence is applicable to clinical specimens [Aesif et al., 2009]. A stepwise description of this procedure is illustrated in Figure 3. The first step in this procedure is to deparaffinize tissue sections, followed by rehydration using a graded series of alcohol. Sections are next permeabilized with 1% Triton X100 in the presence of 40 mM of the thiol blocking agent, N-ethyl maleamide, for 30 min. After three washes in PBS, sections are then incubated with PSSG derivatization buffer containing recombinant Grx1, GSH, NADPH, and glutathione reductase for 20 min, in order to decompose the PSSG bond, leading to a newly formed sulhydryl group. The next step involves incubation with 1 mM biotinylated NEM for 1 hour, in order to label the newly generated SH group. Patterns of PSSG can subsequently be visualized by detection of the biotin moiety with fluorophore-conjugated streptavidin reagents, or anti-biotin antibodies. As a control, representative tissues are subjected to the same procedures, but Grx1 is omitted from the PSSG derivatization buffer [Aesif et al., 2009]. In order to further validate that this method indeed detects S-glutathionylated protein, fully reduced bovine serum albumin (BSA), Cystinylated BSA or S-glutathionylated BSA was added to the Grx1 derivatization mix, and revealed that only the presence of S-glutathionylated BSA competed effectively with Grx1 for de-glutathionylation, leading to a loss of labeling [Reynaert et al., 2006]. Using this methodology we have demonstrated changes in patterns of S-glutathionylation in diverse models of lung disease (Fig. 3) [Aesif et al., 2009], demonstrating the applicability of this technique for the detection of altered S-glutathionylation patterns in diverse disease settings.

SUMMARY

Protein thiols have emerged as cardinal regulators of biological processes, and are critical sites of enzyme regulation through highly regulated and reversible oxidation reactions.

Among these modes of regulation, S-glutathionylation has gained appreciation as a critical event in the regulation of biological processes, and is in turn controlled by several enzymatic pathways. Despite their remarkable potential in controlling (patho)biological processes, analytical tools that enable detection of these thiol oxidative events have lagged behind, in part due to the reversible or labile nature of some thiol oxidations, the common lack of attention to redox changes during tissue disruption and sample processing due to which many biologically relevant redox changes have escaped detection. In addition, reagents to directly detect these oxidative events that can be applied to intact cells or tissues have lacked, although great progress has recently been made in this area [Seo and Carroll, 2009; Seo and Carroll, 2011]. By taking advantage of the catalytic properties of Grx1, we have described here a derivatization method in order to reveal proteins that were S-glutathionylated, and that can be used in fixed cells as well as archival tissues. This method should enable further investigation into the role of S-glutathionylation in diseases, and forms a basis for potential therapeutic intervention aimed at controlling the S-glutaredoxin/glutathionylation redox axis.

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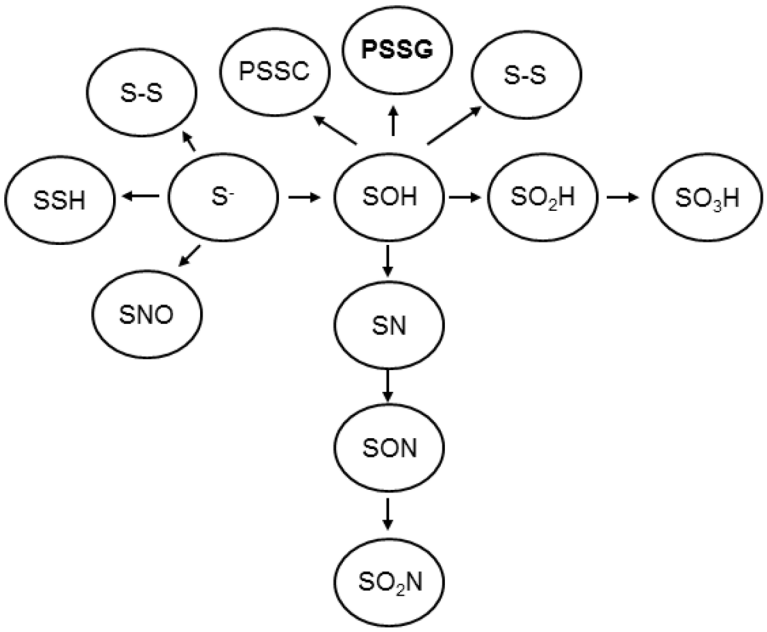

Fig. 1.

A schematic overview of various protein cysteine oxidations. The reactive, low pKa cysteine is shown in the thiolate (S-) state, and can be oxidized in diverse manners. The sulfenic acid intermediate (SOH) is considered a gateway oxidation that can give rise to other oxidations that include S-glutathionylation (PSSG), S-cysteinylation (PSSC), disulfides (S-S), sulfinic (SO_2H), and sulfonic acid (SO_3H) intermediates. SOH can also give rise to sulfenamides (SN), sulfinamides (SON) and sulfonamides (SO_2N). Protein S-nitrosylation (SNO), sulhydration (S-SH) also represent oxidative modifications of reactive protein cysteines that have important roles in the regulation of biological processed. Note that this schematic is an oversimplification. Many conversions between various oxidized states are possible. In many cases the exact oxidation state that gives rise to further oxidations remains unknown.

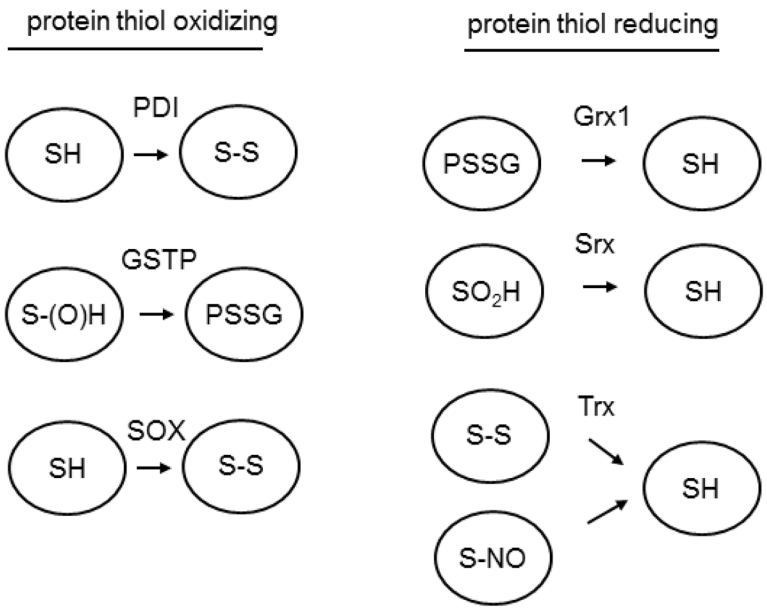

Fig. 2.

Overview of mammalian enzymes that control the oxidation state of protein cysteines. Protein thiol oxidizing: The family of protein disulfide isomerases (PDI), catalyze the formation of disulfide bridges. Sulfhydryl oxidases (SOX), including glutathione peroxidases 7 and 8, ER oxidoreductin 1, quiescin sulfhydryl oxidase, vitamin K epoxide hydrolase, and peroxiredoxin 4 also create disulfide bridges into proteins, and regulate thiol redox homeostasis in the ER. Glutathione S-Transferase P (GSTP) has been implicated in catalyzing S-glutathionylation (PSSG). Protein thiol reducing: mammalian glutaredoxin-1 (Grx1) under physiological conditions catalyzes deglutathionylation reactions restoring the reduced sulhydryl group (SH). Sulfiredoxins (Srx) regenerate 2-Cys peroxiredoxins in the sulfinic state, restoring the SH group. Thioredoxins (Trx) reduce disulfide bonds in proteins to the reduced sulfhydryl state (SH), and also play a role in de-nitosylation reactions (SNO $\rightarrow$ SH). Note that the enzymes listed have also been implicated in regulating alternative oxidation reactions, and that other family members within the same enzyme class can have alternative functions.

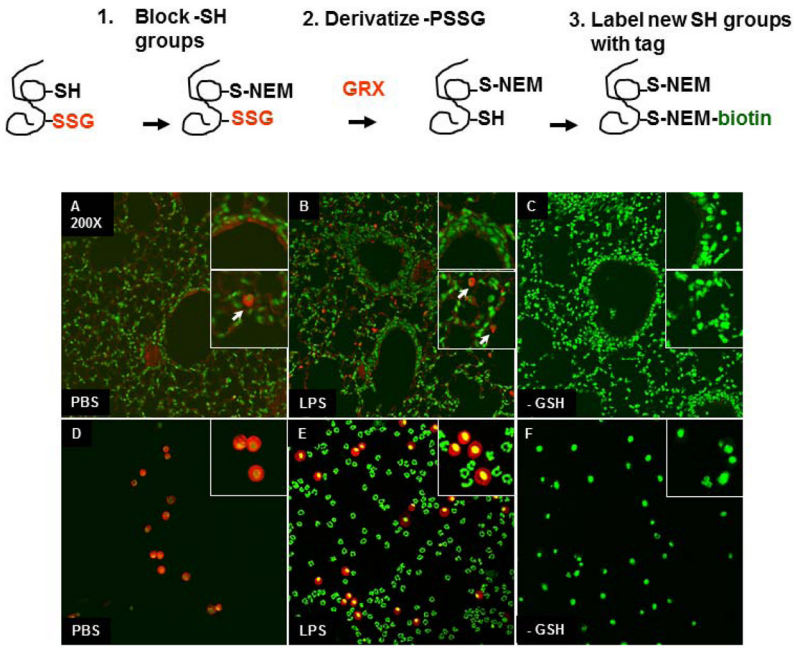

Fig. 3.

Visualization of protein S-glutathionylation *in situ* in cells or tissues following glutaredoxin-1 catalyzed cysteine derivatization. Top: Schematic representation of steps in the procedure. Detailed information is provided in the text and elsewhere [Aesif et al., 2009]. Bottom: In situ analysis of PSSG in lung tissue (A–C, top) or cells isolated from bronchoalveolar lavage fluid (D–F, bottom) from mice following administration of PBS or lipopolysacchide (LPS) to induce an acute inflammatory response. Green staining represents DNA; red staining represents the signal obtained following Grx1-catalyzed derivatization and reflects PSSG. As a control GSH was omitted from the reaction mix (-GSH) and similar results were obtained in the absence of Grx1. Note the marked PSSG reactivity in mononuclear cells with an appearance of macrophages, and absence of detectable PSSG in neutrophils (bottom). Magnification = 200X. Figure 3 is reprinted from The American Journal of Pathology, Vol. 175, No. 1, Scott W. Aesif, Vikas Anathy, Marije Havermans, Amy S. Guala, Karina Ckless, Douglas J. Taatjes, and Yvonne M.W. Janssen-Heininger, *In Situ* Analysis of Protein S-Glutathionylation in Lung Tissue Using Glutaredoxin-1-Catalyzed Cysteine Derivatization, Pages No. 36–45, 2009, with permission from Elsevier.