



Review Article

Redox regulation of protein damage in plasma



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ABSTRACT

The presence and concentrations of modified proteins circulating in plasma depend on rates of protein synthesis, modification and clearance. In early studies, the proteins most frequently analysed for damage were those which were more abundant in plasma (e.g. albumin and immunoglobulins) which exist at up to 10 orders of magnitude higher concentrations than other plasma proteins e.g. cytokines. However, advances in analytical techniques using mass spectrometry and immuno-affinity purification methods, have facilitated analysis of less abundant, modified proteins and the nature of modifications at specific sites is now being characterised. The damaging reactive species that cause protein modifications in plasma principally arise from reactive oxygen species (ROS) produced by NADPH oxidases (NOX), nitric oxide synthases (NOS) and oxygenase activities; reactive nitrogen species (RNS) from myeloperoxidase (MPO) and NOS activities; and hypochlorous acid from MPO. Secondary damage to proteins may be caused by oxidized lipids and glucose autooxidation.

In this review, we focus on redox regulatory control of those enzymes and processes which control protein maturation during synthesis, produce reactive species, repair and remove damaged plasma proteins. We have highlighted the potential for alterations in the extracellular redox compartment to regulate intracellular redox state and, conversely, for intracellular oxidative stress to alter the cellular secretome and composition of extracellular vesicles. Through secreted, redox-active regulatory molecules, changes in redox state may be transmitted to distant sites.

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Abbreviations: BH4, tetrahydrobiopterin; CRP, C-reactive protein; COX, cyclo-oxygenase; ER, endoplasmic reticulum; ERO1, endoplasmic reticulum oxidoreductin 1; EV, extracellular vesicles; FX1, factor XI; GPI, glycoprotein 1; GPX, glutathione peroxidase; GRX, glutaredoxin; GSH, glutathione; MIRNA, microRNA; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; O₂⁻, superoxide anion radical; ONOO⁻, peroxynitrite; PDI, protein disulphide isomerase; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive nitrogen species; Trx, thioredoxin; VWF, von Willebrand factor; XO, xanthine oxidase

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Introduction

Plasma proteins perform a range of important physiological functions such as maintaining homeostatic blood volume, transporting other molecules for delivery at distant sites, through to regulating endocrine systems and inflammatory responses. With half-lives ranging from minutes to a month (Table 1) and with biosynthetic and turnover rates decreasing during ageing, their potential to accumulate damage differs markedly by protein and over time [1,2]. Consequently, modifications to plasma proteins may exert a range of diverse effects according to the sites of damage and are reported to increase in frequency with age, acute and chronic diseases. Therefore, modified proteins have the potential to serve as important biomarkers and may in turn signpost aetiological mechanisms [3]. An improved understanding of factors that influence the steady state concentrations of damaged proteins is important for evaluating their sensitivity as biomarkers and also their potential as targets for therapeutic interventions that prevent or repair or modifications. The focus of this review is on the role of redox regulation of steady state protein damage in plasma.

In reviewing the redox regulation of protein damage in plasma, we will consider (1) errors introduced in biosynthesis e.g. during ER stress that affect glycosylation, folding and secretion: (2) redox control of myeloperoxidase (MPO), NAPH oxidase isoforms (NOX), nitric oxide synthases (NOS), xanthine oxidase (XO) which increase protein exposure to reactive oxygen and nitrogen species (ROS, RNS) in the plasma and result in chlorination, nitration, nitrosylation, chlorination, methionine oxidation, disulphide formation, HNE-protein adducts: and (3) regulation of hepatic and macrophage receptors, extracellular reducing enzymes and proteins such as protein disulphide isomerase (PDI), thioredoxin1 (Trx1), peroxiredoxins (Prx) and oxidoreductases that affect steady state level of plasma protein damage.

Historically, the proteins analysed most frequently for damage were the more abundant plasma proteins (e.g. albumin and immunoglobulins) occurring at up to 10 orders of magnitude higher concentrations than other proteins found in plasma; more recently improved purification methods and higher sensitivity mass spectrometry techniques have enabled less abundant proteins to be examined [4].

Redox regulation in protein synthesis

There is little protein specificity for ROS and RNS, with reactions often proceeding at diffusion controlled rates, such that the proteins most likely to be damaged by ROS are those in closest proximity to their sites of production and at the highest concentrations.

During protein synthesis, secretory and membrane proteins co-translationally enter and are folded in the endoplasmic reticulum (ER) and Golgi. Oxidative disulphide bond formation and glycosylation facilitate correct protein folding prior to transport to the plasma

membrane for export; misfolded ER proteins are recognised and unfolded by ER resident reductases and chaperones before undergoing retrotranslocation to the cytosol [5].

Oxidative maturation is achieved by highly regulated enzymatic transfer of two electrons [6]. The first conserved ER-resident oxidase in the pathway to be identified that generates disulphide at the expense of reducing oxygen is oxidoreductin 1 (Ero1) which occurs in two discretely regulated and distributed forms, alpha and beta; however, in contrast to observations in yeast, double Ero1 knockout animals show little phenotype. Indeed, normal ER redox conditions can be established after a strong reductive challenge, although this occurs more slowly than in wild-type cells, suggesting a role for other oxidative enzymes in disulphide formation [7]. One candidate family is the protein disulphide isomerases (there are 20 reported family members in mammalian cells) that include PDI, glutathione peroxidase (GPx) 7, GPx8 and which interact with Ero1alpha [8]. Ero1alpha activity is inhibited by an intramolecular disulphide switch between the active-site Cys94 and Cys131 and is re-activated by available reduced PDI. In support of this regulatory mechanism, overexpression of the mutant Ero1alpha-Cys131Ala which does not have a disulphide switch, leads to ER overoxidation [9]. In one of the first studies to identify specific oxidised thiol sites on intracellular proteins, using methoxypolyethylene glycol 5000 maleimide, Herzog-Appenzeller et al. showed that PDI is found in two semi-oxidised forms suggesting that either domain in human PDI can catalyse substrate oxidation and reduction [10]. Both isoforms of Ero1 facilitate the propagation of disulphides via PDI to nascent proteins and hence are crucial for oxidative maturation [11] in a process that is modulated by the glutathione (GSH)-oxidised GSH (GSSG) redox pair [12]. Other redox regulated enzymes that have been implicated in control of protein folding include; (a) Prx4 which can use luminal hydrogen peroxide to oxidise PDI and thereby favour oxidative folding but limit oxidative stress; and (b) vitamin K epoxide reductase in cooperation with membrane-bound Trx-like redox partners [13]. The extent of redundancy in the pathways for oxidative protein folding supports the importance of effective redox control in the biosynthesis of secreted proteins.

There are very few examples of loss of redox control in the ER which impact on the secretome. An early study by Lodish used DTT to explore the effect of a strongly reducing environment on the secretion of different proteins and found that only secretion of those with disulphide bonds was reversibly inhibited by DTT [14]. In contrast, a recent study describes that in astrocytes overexpressing mutant SOD1, total protein secretion was decreased although increased mutant SOD1-containing exosome release was observed, possibly to prevent intracellular aggregate formation [15]. It remains to be determined how the conventional ER secretory pathway is affected by SOD1 mutant and whether this is due to excess ROS. The extent to which exosome and microparticle (extracellular vesicles; EV) formation can influence protein damage or transport modified proteins is unknown, however, a few intriguing reports suggest that EV can induce redox signalling at distant sites [16–18] and that their

Table 1
Major plasma protein characteristics.

Plasma protein	Normal level	(%)	Function	Half-life
Albumin	3.5–5 g/dl	60	Create oncotic pressure Carry other molecules	17d
Immunoglobulins	1–1.5 g/dl	18	Acquired immune response	19–24 d in healthy subjects
Fibrinogen	0.2–0.45 g/dl	4	Blood clotting	3.5–5.5 d
α -globulins	0.15–0.35 g/dl	3	Anti-trypsin	62 h
	0.03–0.2 g/dl	2	Haptoglobin	8 h
β globulins	0.2–0.36 g/dl	4	Transferrin,	< 24 h
	0.06 g/dl	1	High Density Lipoprotein	
	0.1 g/dl	2	Low density lipoproteins	
Hormones (e.g. norepinephrine)	0.6 NM		Promote stress response	2.5 min

secretion may be influenced by redox state [19]. This promising field of research may reveal a novel mechanism of redox signaling.

Discrete plasma protein glycosylation patterns have been described during disease and ageing. These may arise from synthetic errors in B cells e.g. during rheumatoid arthritis and also from post-translational modifications by ROS [20–23]. Few studies describe the effects of glycoform modifications as they are both complicated to design and to analyse. Using a series of glycosidases to prune the glycoform, the effect of oligosaccharide structure has been described for IgG1, with the glycoform changing effector functions of the molecule in binding complement and Fc receptors [24]. In a different approach, the function of C-reactive protein (CRP) with different glycosylation patterns that was isolated from patients with infectious disease was examined; patient CRP caused greater erythrocyte fragility which the authors attributed to the glycosylation pattern and may explain in part the anaemia associated with tuberculosis and leishmaniasis [25].

Cullen et al. explored the effect of oxidised LDL-loaded macrophages on membrane protein glycosylation and described a decrease in alpha-1,3-fucosyltransferase enzyme activity and sialyl lewis expression which may reduce macrophage binding to the selectin receptor, modulating homing in atherosclerotic vessels [26]. Direct evidence for the involvement of ROS in regulating glycosylation has been provided in TNFalpha-activated leukocytes lacking the NOX component, gp91 phox, p47 phox or inhibited with tiron. After NOX inhibition, rather than an increase in core 2 beta-1, 6-N-acetylglucosaminyltransferase activity induced by TNF, transferase activity was reduced by NOX inhibition and binding to endothelial cells was also inhibited [27]. This suggests a role for redox regulation of membrane protein glycoforms. From the aforementioned evidence, loss of the native protein glycoform can be elicited directly by oxidative damage or via redox regulation of oligosaccharide synthesis; irrespective of the cause, protein function is often affected by altering the glycoform.

Redox regulation of protein damage inducers

The molecular fingerprint of damage to major plasma proteins includes amino acids modified by chlorination, nitrosylation, oxidation, nitration, crosslinking (disulphide and Schiff base) formation, glycation, dialdehydic lipids such as malondialdehyde and 4-hydroxynonenal [28–35]. The mechanisms underlying these modifications have been considered extensively elsewhere and are not considered here [36]. The damaging reactive species principally arise from ROS produced by NOX, NOS and oxygenase activities; RNS from MPO and NOS activities; and hypochlorous acid from MPO. Instead, our focus is on redox regulatory control of those enzymes which produce reactive species.

Redox control of NAPH oxidase isoforms (NOX)

Probably the most important source of the ROS, superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), derive from NOX 1–5 and Duox1/2 [37]. ROS from these enzymes are produced at vastly different rates and concentrations to effect signalling and bacterial killing. The majority of NOX forms release $O_2^{\cdot-}$ to the extracellular face and therefore have the potential to cause bystander damage to plasma proteins. The NOX family is widely distributed and by virtue of its potential to induce widespread damage, its activity is carefully regulated. PDI is a key regulatory enzyme of NOX. It controls angiotensin II-mediated ROS generation and Akt phosphorylation in vascular cells, being co-located with oxidase subunits probably playing a role in subunit assembly or trafficking [38]. Similarly in macrophages, close association between PDI and the p22phox NADPH oxidase subunit was shown

by confocal co-localization and co-immunoprecipitation [39]. In neutrophils, the role of PDI in NOX assembly was also demonstrated, where oxidised PDI increases, and reduced PDI decreases membrane NADPH oxidase complex activity. Cytosolic PDI exhibited thiol-dependent association with p47(phox) but following activation did not accumulate in membranes. In contrast, oxidised PDI was detected in the membrane suggesting a role in NOX organisation according to its redox state [40]. Further investigation of PDI binding partners revealed functional associations with small GTPases Rac1 and RhoA and their regulator RhoGDI and suggested that PDI supports NOX1 activation in vascular smooth muscle cells [41]. In endothelial cells, many molecular and physical stimuli can activate NOX signalling which converge on a redox regulatory mechanism through Ras GTPases that contain a redox-sensitive cysteine (X) in the conserved NKXD motif and are susceptible to single electron reduction. Similarly, Rho GTPases that contain a redox-sensitive cysteine at the end of the conserved phosphoryl-binding loop motif (GXXXXG[S/T]C) may be activated by single or two electron cysteine oxidation [42,43].

The assembly of NADPH oxidase subunits is also driven by lipid raft formation, membrane macrodomains enriched in sphingolipid and cholesterol [44,45]. We have shown that intracellular glutathione concentrations are lower in neutrophils from patients with chronic periodontitis and thiol-regulated acid sphingomyelinase activity is increased, driving raft formation and is associated with an increase in circulating plasma protein oxidation in periodontitis [45,46]. Intracellular GSH redox state and the ability to drive an effective adaptive response to regenerate GSH after its depletion via Nrf2 activation are important regulatory elements during NOX activation. GSH may be used as reducing equivalents by PDI to inactivate NOX, can exist at different redox states within a cell according to compartment and interestingly can be affected by extracellular redox state affording “outside-in” control of activation [47–49].

Redox control of nitric oxide synthases (NOS)

Nitric oxide (NO) is generated by three NOS isoforms at varying rates and concentrations according to its role in cell signalling or bacterial killing. It can diffuse through membranes for up to 6 μ m from its site of production facilitating distant effects. NOS are highly regulated through a number of discrete mechanisms including transcriptional control (redox and NFkB for iNOS/NOS2) calcium, calmodulin and tetrahydrobiopterin (BH4) availability. NO can mediate direct and reversible nitrosylation of thiol moieties important for signalling and so is considered protective against the effects of ROS which may oxidise thiols to higher, irreversible oxidation states. On the other hand, when produced at higher concentrations and combined with $O_2^{\cdot-}$, NO produces the potent oxidant peroxynitrite, ONOO $^-$. As described for NOX, the small GTPase Rac, also regulates constitutive NOS1 and 3 found in neurons and endothelial cells respectively. Cellular NO and $O_2^{\cdot-}$ production increase or decrease in a coordinated fashion through Rac as a common control element [50].

NOS2 is located in peroxisomes and the cortical cytoskeleton is induced by ROS and NFkB during inflammation and tissue injury as part of the host defence and healing mechanisms. NOS2 induction results in NO release into the oxidative microenvironment, results in peroxynitrite formation and can contribute to tyrosine nitration in plasma proteins [51].

NOS3 was first reported in endothelial cells and is calcium/calmodulin-dependent and normally produces NO for signalling. However, during oxidative stress, BH4 is depleted, and NADPH oxidation is uncoupled from NO generation, leading to production of $O_2^{\cdot-}$; uncoupling is also driven by phosphorylation of Ser-1177 by Akt such that $O_2^{\cdot-}$ generation is Ca^{2+} -independent [52–54]. The adipokine, resistin, which is considered important in insulin

resistance, diabetes and cardiovascular disease, can also inhibit NO production from NOS3 in endothelial cells and an increase in ROS albeit in this case from loss of NOS3 mRNA stability and mitochondrial ROS, respectively [52]. Disruption of NOS3 is likely to result in loss of effective NO signalling and increased plasma protein oxidation. In contrast, upregulation of NOS3 by Nrf2 and NF- κ B activation by isoflavones may increase NO bioavailability offering a mechanism whereby dietary phytoestrogens may modulate the redox environment and protein damage [55].

Regulation of lipoxygenase, xanthine oxidase and MPO

Cyclooxygenase (COX)-2 and 5-lipoxygenase, which synthesise the inflammatory mediators, prostaglandins and leukotrienes, from dietary or membrane-originated AA via phospholipase A2 activity also produce ROS as byproducts [52]. While the effects of COX-2 dependent biosynthesis of resolution phase lipid mediators are fine-tuned by micro RNAs (miRNA) [56], little is known of regulators of ROS production. While there is evidence to suggest that dietary modulation of miRNA expression may affect COX-2 activity, this work is still in its infancy.

Xanthine oxidase (XO) is a potentially important source of superoxide in the vasculature during hypoxia and depletion of ATP, when xanthine dehydrogenase activity is converted to oxidase activity following thiol oxidation and the enzyme degrades purines and produces superoxide.

MPO is found in azurophilic granules of neutrophils and activity is regulated only by availability of substrate hydrogen peroxide and compartmentalization. It can mediate chlorination and nitration of proteins at inflammatory sites [33,57]. Owing to the unique source and chemistry of MPO products, there have been several successful approaches to limit MPO-driven oxidative damage [58,59].

Redox regulation of repair and receptor-mediated clearance for damaged proteins in plasma

The presence of proteins that have been damaged by reactive species in the circulation is not only due to excess production of reactive species but also due to inadequate repair or removal of damaged molecules. Only a limited number of damages are repairable, including disulphide bonds, sulphenic and sulphonic acids, nitrosylation and methionine sulphoxides. There are limited examples of protein repair occurring in fluid phase, whereas several proteins are reduced on the cell surface by exported oxidoreductase enzymes such as PDI and cofactors including Ero1, Trx and Prx. No reports of extracellular methionine sulphoxide reductase were found.

Hepatic and phagocytic receptor-trafficked clearance mechanisms are the major routes for removal of damaged proteins and these are subsequently degraded intracellularly by the proteasome. Extracellular matrix proteins accumulate damage over their life-time and collagens can become heavily glycosylated, undergoing extensive browning and elasticity loss, during diabetes.

Receptor mediated clearance

The liver serves a variety of functions including the synthesis and removal of many plasma proteins. Protein clearance is most likely mediated by Kupffer cells, specialised liver macrophages which carry receptors for asialoglycoproteins. Loss of sialic acid from glycoproteins may contribute to enhance antigenicity, loss of tolerance and development of “autoimmune” responses hence rapid removal is important [60,61]. Liver function declines with age and also after ethanol exposure due to impaired binding of asialoglycoproteins by their receptors [62]. When combined with

genetic polymorphisms in HLA antigens, the risk for autoimmunity is increased in older adults and younger adults who experienced chronic hepatitis C as children [63].

Another class of receptors that mediate clearance of circulating damaged proteins in plasma are scavenger receptors expressed on Kupffer cells [64] and macrophages e.g. CD36 [65]. CD36 binds to oxidised and hypochlorite-modified apoproteins within HDL and LDL and may elicit a range of downstream signals according to co-receptor involvement. The nature of the epitopes recognised by CD36 are not well-defined but are more likely to be molecular patterns of hydrophobic amino acids caused by exposure of hydrophobic domains following damage. Once internalised, the damaged proteins will be degraded in lysosomes.

Damaged protein repair

The oxidoreductases and Trx1, can be exported through the leaderless secretory pathway, exerting a range of effects on T cells, B cells and fibroblasts from growth arrest to autocrine activation and can also be found associated with plasma membranes where it is probably anchored through palmitoylation [66]. We have shown that Trx1 trafficking to the membrane is decreased when intracellular GSH is depleted and hence the intracellular redox state may influence the extracellular environment [67]. Trx1 is an effective reductant of disulphide bonds in plasma and on the cell surface.

One notable example of oxidised protein repair in plasma is in the regulation of tissue factor (TF) activity by secreted Trx1 [68]. Enhanced tissue factor activity increases thrombus risk. This can be decreased through disulphide reduction by NADPH, Trx1 and thioredoxin reductase (TxR) and results in reversible association of Trx1 with TF in human serum and plasma samples so interfering with factor 7a binding to TF and inhibition of the coagulant cascade. However, others have debated the importance of redox control for activation of TF and suggest a role for PS exposure and suggest further study is necessary [69,70]. The activity of a related clotting factor is also increased by reduction; circulating Factor XI (FXI) exists as a dimer with disulphide bonds which can be reduced by Trx1 and PDI. The activation of reduced FXI by thrombin, FXIIa or FXIa was significantly increased compared to non-reduced FXI thereby promoting an effective clotting response [71].

In another example, this time in close association with T cell membranes, IL-4 activation and binding to receptor is achieved through membrane associated Trx and PDI with TxR serving as the electron donor [72]. Full length Trx1 on endothelial cells acts as an inhibitor of C5 convertase deposition, so preventing formation of complement component C5a and the membrane attack complex in a redox-dependent manner [73]. However, the truncated form of Trx1 was a potent activator of complement and highlights the importance of understanding the form of Trx1. Other disulphide bonds on leukocytes that are labile and can be reduced by Trx with PDI and thiol reductase, include mouse T cell CD132. When reduced, IL-2 dependent signalling was inhibited but it is unknown whether this same effect is observed in human cells, where oxidation of cell surface proteins is associated with suppression of activity [74]. Related to Trx1, PDI and glutaredoxin (Grx1) activity is required for T cell entry of HIV1 after binding to surface CD4 through reduction of intramolecular disulphides in the envelope protein gp120. To date, Grx1 does not appear to be widely used as a reducing enzyme at the T cell surface so may be a druggable target to prevent HIV-1 entry [75].

In addition to their role in thrombosis, platelets are increasingly recognised as important players in inflammatory responses, secrete EVs and provide an important platform for oxidised protein repair. For example, the plasma protein beta(2)-glycoprotein I (GPI) circulates in an oxidised state and is susceptible to reduction at Cys288–Cys326 disulphide in domain V by Trx1 and

PDI on the platelet surface. Reduced beta(2) GPI shows increased binding to von Willebrand Factor (vWF) and in turn increased platelet adhesion to activated vWF [76].

Surface adhesion molecules, integrins, are important for cell to cell interactions and frequently undergo post-translational modifications on activation that increase their binding to their cognate receptors. Redox-controlled remodeling of the exofacial domains of alpha(Ib)beta(3) integrin on platelets by exofacial PDI with Ero1alpha to reoxidise the PDI active site, increases receptor activity and fibrinogen binding [77,78]. Other proteins on the cell surface regulated by PDI include ADAM17 and on platelet surface that are redox sensitive include thiol isomerase enzymes e.g. ERp57 which respond to changes in the extracellular redox environment and their impact on platelet activation which along with other family members deserves further study as potential targets against thrombus formation [79–81]. Repair of oxidised thiols maintains signalling through a number of different receptors and although not strictly damaged, the careful control of reduction and oxidation by close association with oxidoreductases prevents irreversible hyperoxidative damage occurring.

Another important antioxidant-like enzyme family with reducing activity is the peroxiredoxin family. Prx6 translocates to the neutrophil surface and is required for respiratory burst activation, however, mutant proteins (C47S) and (S32A) lacking reductase and phospholipase activity remained active for respiratory burst activation and therefore the role of surface Prx6 remains unknown [82].

Conclusion

This review of redox regulation has considered processes that control the extent of damaged proteins in plasma and regulation of enzymes that contribute to the concentrations of reactive species that can cause damage. It has reviewed the manner in which the extracellular redox compartment can regulate intracellular pathways, and conversely, how intracellular oxidative stress may alter the cellular secretome and extracellular protein damage.

However, redox dysregulation and reactive species do not just beget further reactive species but also drive antioxidant protein expression. It is beyond the scope of this review to cover the wealth of literature on redox regulation of antioxidant enzymes regulated by NFkB such as MnSOD or Nrf2 (e.g. GSH peroxidase); the redox-regulated antioxidant response offers the possibility of controlling the extent of reactive species available to damage proteins and to restore the intracellular redox potential. The epigenetic control of adaptive responses promises some interesting opportunities for interventions that might reverse de-/acetylation and de-/methylation and restore redox homeostasis through Nrf2 signalling. The potential for redox signaling through circulating EV suggests an exciting connection to distant sites and potential for modulation by antisense miRNA. The existence of a regulatory antioxidant loop is critical for health and failure to elicit an adaptive response may underlie a protein damage in a number of chronic inflammatory diseases and ageing.

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