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Hydrogen peroxide as a damage signal in tissue injury and inflammation: Murderer, mediator, or messenger?

Albert van der Vliet and Yvonne M. W. Janssen-Heininger

Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405, USA

Abstract

Tissue injury and inflammation are associated with increased production of reactive oxygen species (ROS), which have the ability to induce oxidative injury to various biomolecules resulting in e.g. protein dysfunction or cell death. However, recent observations indicate that formation of hydrogen peroxide (H_2O_2) during tissue injury is also an essential feature of the ensuing wound healing response, and functions as an early damage signal to control several critical aspects of the wound healing process. Because innate oxidative wound responses must be tightly coordinated to avoid chronic inflammation or tissue injury, a more complete understanding is needed regarding the origins and dynamics of ROS production, and their critical biological targets. This Prospect highlights the current experimental evidence implicating H_2O_2 in early epithelial wound responses, and summarizes technical advances and approaches that may help distinguish its beneficial actions from its more deleterious actions in conditions of chronic tissue injury or inflammation.

Keywords

Reactive oxygen species; NOX/DUOX; redox signaling; cell migration; chemotaxis; cysteine

TISSUE INJURY, WOUND RESPONSES, AND INFLAMMATION – INVOLVEMENT OF REACTIVE OXYGEN SPECIES

Throughout life, each organism must regularly cope with various types of injury, and has therefore evolved efficient response mechanisms to sustain such injury and initiate appropriate wound response mechanisms, to maintain or restore tissue integrity and function. Basic wound healing mechanisms are highly conserved, and possess many features that are similar to developmental processes, and rely on common initial damage signals that induce early wound responses, such as cell shape changes and recruitment of immune cells. Ineffective wound responses lead to increased risk of potentially damaging infections, and inappropriate control of these wound responses is commonly associated with chronic inflammation and disease, e.g. due to fibrotic scarring and defective tissue architecture and function, and may also contribute to cancer development, since tumors display many features of wounds that fail to heal. Indeed, most chronic diseases can be viewed as

conditions associated with a failure of normal repair processes. Therefore, a firm understanding of the fundamental mechanisms of wound healing mechanisms is essential in appreciating the basic molecular mechanisms of life and disease.

A wealth of evidence supports the idea that tissue injury is associated with oxidative stress, resulting from either external sources or through endogenous production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and related products, largely due to ongoing inflammatory processes [1, 2]. Indeed, studies in injury models in various organisms have shown increased production of ROS, both after acute wounding as well as in chronic regenerating wounds [3, 4]. Over the years, consensus has been reached that ROS actively contribute to chronic tissue injury or inflammation, due to their ability to induce irreversible oxidation of biological molecules of all classes (proteins, lipids and DNA), and observations that ROS-detoxifying antioxidants can minimize tissue injury and improve wound healing. However, contrasting this general view is a recently emerging concept that ROS are not merely injurious, but can also control many biological processes that are critical in tissue maintenance, defenses against infection, and regulation of inflammatory processes, through redox signaling mechanisms. In this regard, several intriguing studies over the past several years established that early production of ROS in response to tissue injury is in fact critically important in appropriate wound healing. Studies in several model organisms have indicated that rapid production of H_2O_2 in response to wounding is responsible for minimizing infection risk, activating epithelial signaling pathways involved in epidermal regeneration, and recruiting neutrophils and other leukocytes to the wound site, all cardinal features of the overall wound healing response [4-7]. Analogous findings have also been obtained in mammalian models of wound healing, such as during cutaneous injury where H_2O_2 production in dermal wounds was found to contribute to wound angiogenesis and closure [3], and in our recent studies demonstrating a critical role for H_2O_2 production in pulmonary epithelial wound responses and enhanced epithelial cell migration as a critical early step in epithelial regeneration in the respiratory tract in response to injury [8-10]. Nevertheless, much still remains to be learned about the precise molecular cues that control H_2O_2 production, as well as the cellular or extracellular mechanisms through which it promotes wound responses. This Perspective will not attempt to comprehensively review the various roles of ROS in tissue injury and inflammation, but will rather highlight the burgeoning evidence demonstrating the critical importance of H_2O_2 as an early damage signal in wound responses, and discuss technical approaches that are needed understand the principal mechanisms in these oxidative wound responses, and will be helpful in development of therapeutic strategies that exploit these beneficial oxidative wound responses or prevent the potential adverse role(s) of ROS in conditions in impaired wound healing or in chronic inflammation and injury.

H_2O_2 AS A CONSERVED DAMAGE SIGNAL IN INJURY RESPONSES

Cellular responses to injury are initiated by highly conserved immediate damage signals that mediate early, transcription-independent wound responses and contribute to activation of various (growth factor) ligands and receptors to activate signaling pathways and gene transcription. While the overall molecular events involved in wound healing processes are highly complex and multifactorial, aggregate recent findings point to the common

importance of three inter-related diffusible molecular triggers as the earliest damage signals in wound responses [6, 11]. The first of these is Ca^{2+} , which is kept at very low steady-state cytosolic levels (<100 nM) compared to mM extracellular concentrations, thus creating a steep Ca^{2+} gradient across the plasma membrane. Perturbances in this gradient are easily provoked by many diverse chemical or mechanical triggers, which typically result in transient increases in intracellular Ca^{2+} by Ca^{2+} influx through e.g. activation of voltage-gated Ca^{2+} channels or transient receptor potential (TRP) channels or through release from internal Ca^{2+} stores (by activation of G-protein coupled receptors at the cell surface) (Fig. 1). The precise mechanism of Ca^{2+} increase is highly stimulus-dependent and not always completely understood, but abundant experimental evidence indicates that such Ca^{2+} increases are critically involved in cellular wound responses [11]. Elegant studies in cultured epithelial cells or intact organisms (e.g. zebrafish) using Ca^{2+} -specific fluorescent sensors indicate the occurrence of rapid Ca^{2+} waves or flashes within seconds after wounding, and blockade of Ca^{2+} entry or chelation of intracellular Ca^{2+} typically impairs wound responses [5, 6, 12]. The mechanisms by which such Ca^{2+} signals promote wound responses are diverse, and include activation of protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase, calpains, as well as other Ca^{2+} -activated targets [11].

Purinergic molecules such as ATP constitute a second highly conserved danger signal, being highly abundant intracellularly in healthy cells (estimated to be ~100 mM in case of ATP) compared to dramatically lower extracellular concentrations (<10 nM; thus constituting a 10^6 -fold transmembrane gradient), due to tight control of cellular ATP release and the presence of ATP degrading ectoenzymes on the cell surface. Cellular release of ATP is readily provoked by mechanical stimuli including shear stress as well as various chemical and biological triggers, either through vesicular release pathways or passive release to connexin or pannexin hemichannels, generating extracellular ATP concentrations sufficient to act as autocrine or paracrine transmitters through activation of purinergic P2Y and P2X receptors on the cell surface and stimulation of cellular signaling pathways that control wound responses [13]. Activation of P2Y receptors (G protein-coupled receptors) and P2X receptors (which act as ligand-gated ion channels) can induce intracellular Ca^{2+} increases as a part of their signaling mechanism [13], and conversely, Ca^{2+} -dependent signaling mechanisms can also contribute to promoting ATP release [11], thus illustrating a close and reciprocal relationship between these two danger signals.

A number of studies have demonstrated that increases in intracellular Ca^{2+} as well as extracellular ATP are causally linked to cellular or extracellular production of ROS such as H_2O_2 , often due to the activation of NADPH oxidases. Seven mammalian NADPH oxidase (NOX) isoforms exist, which are widely distributed and are activated depending on Ca^{2+} -mediated signaling, especially in isoforms containing intrinsic Ca^{2+} -binding EF-hand motifs within one of their internal domains, such as NOX5 or the DUOX enzymes [14]. Recent studies in e.g. zebrafish models of tail fin injury have demonstrated that rapid Ca^{2+} flashes in response to injury are directly responsible for early H_2O_2 production, which originates from their single DUOX NADPH oxidase homolog [6, 12]. Similarly, studies in mammalian cell systems as well as plants have shown that ATP (through P2Y receptor stimulation) can promote cellular ROS production by activation of NADPH oxidases [14]. Indeed, studies from our laboratory demonstrated rapid wound-induced H_2O_2 production in bronchial

epithelial cells due to activation of its major NADPH oxidase homolog DUOX1, in response to ATP-dependent signaling through P2Y purinoceptors [8, 9]. Cellular production of H₂O₂ (or related ROS) appears to be a common result of concerted actions of Ca²⁺-dependent signaling as well as ATP-dependent purinergic signaling, which promotes activation of e.g. protein kinases A or C that also contribute to NADPH oxidase activation [14]. Because of the various biological actions of H₂O₂, which will be discussed in the following sections, cellular production of H₂O₂ can be viewed as a third early damage signal, that is closely associated with the other two damage signals, Ca²⁺ and ATP. This intricate association is further exemplified by findings that H₂O₂ production can itself contribute to Ca²⁺ influx as well as ATP release (e.g. [14]), indicating that the precise relationships between these danger signals are complex and reciprocal.

It is apparent from the above that production of H₂O₂ or related ROS is an integral and conserved component of injury-related cell responses and functions as an important damage signal. As will be discussed in the following sections, various functional actions have been ascribed to H₂O₂ in the context of wound responses, ranging from direct antimicrobial defense (“murderer”) to autocrine or paracrine cell signaling and gene regulation (“mediator”) and chemotactic properties that regulate microbial invasion or recruitment of inflammatory cells such as neutrophils (“messenger”). The precise biochemical mechanisms by which H₂O₂ exerts these actions is not always fully understood, as the biological actions of H₂O₂ are not mediated by classical receptor/ligand interactions, but rather by its ability to oxidize susceptible molecular targets with some specificity. The recent development of experimental approaches to identify such molecular oxidant-sensitive targets has begun to provide important insights into the general mechanisms of H₂O₂-dependent redox signaling targets, and their application will be critical to establish the central mechanisms in H₂O₂-dependent injury responses as well as their regulation by other external factors.

H₂O₂ AS A MURDERER: ANTIMICROBIAL AND REPELLANT PROPERTIES

The deliberate production of H₂O₂ and related ROS by phagocytic cells has been widely recognized as playing an important role in antimicrobial host defense, perhaps best illustrated by the fact that genetic deficiency of the phagocyte NADPH oxidase, the key oxidant-producing enzyme system, results in enhanced susceptibility to bacterial or fungal infection [15]. However, various NOX homologs are also expressed in non-phagocytic cell lineages including epithelial cells of the respiratory or intestinal tract, where they were originally presumed to have similar antimicrobial function. For example, respiratory and intestinal epithelial cells express the dual oxidases DUOX1 and DUOX2 at their apical surface, and can generate luminal H₂O₂ production as a potential host defense mechanism [14]. An important component of such oxidative host defense function is the local presence of lactoperoxidase that is secretion within the airway or intestinal lumen to utilize DUOX-derived H₂O₂ to generate more potent antimicrobial oxidants, such as HOSCN [16, 17] (Fig. 2). More direct evidence for DUOX in innate intestinal antimicrobial defense has been provided by studies in various non-mammalian organisms, including *Drosophila*, *C. elegans* or zebrafish, in which their single DUOX gene appears to be directly responsible for oxidative antimicrobial activity and survival after microbial infection [18, 19]. A critical aspect of this DUOX-dependent host defense mechanism includes the fact that pathogen-

induced mechanisms also lead to induction of DUOX expression [20]. The importance of mammalian DUOX1 and DUOX2 in antimicrobial host defense is less well established, and appears to involve isoform-specific functions depending on the nature of the infection, illustrated by pathogen-specific induction of DUOX1 or DUOX2 in mammalian epithelia [14]. Intriguing recent studies also indicate that DUOX is selectively engaged in antimicrobial responses to opportunistic bacteria while allowing for harmonious colonization of commensal microbiota without DUOX activation, depending on specific DUOX activation by pathogen-dependent products such as uracil [20]. The relative roles of mammalian DUOX1/2 in such selective pathogen-dependent responses are at present unknown.

Although these various lines of evidence indicate a critical role for DUOX in innate antimicrobial defense, it is less clear whether it supports direct H_2O_2 -dependent killing or alternative antimicrobial mechanisms. Contrary to confined H_2O_2 production in activated phagocytic cells within the phagosome, H_2O_2 production at mucosal surfaces occurs within a much large volume (5-20 μm within the respiratory tract, and even higher in the gut), and is unlikely to reach sufficient concentrations to induce direct bacterial killing, which typically requires near mM concentrations [21]. Because the inter-relationship between bacteria and H_2O_2 is most likely much more complex, since bacteria themselves can also generate H_2O_2 and express H_2O_2 -detoxifying enzymes such as catalase, it has been proposed that DUOX-derived H_2O_2 maintain epithelial sterility not by direct oxidative killing but rather by creating H_2O_2 gradients that repel bacteria from the epithelial surface to prevent epithelial injury. This is consistent with the fact that bacteria exhibit negative chemotaxis in H_2O_2 gradients [21], and may also help clarify the more selective involvement of DUOX in preventing colonization by opportunistic pathogens without disturbing commensal mucosal bacteria [20]. This concept may also apply to LPO-dependent production of HOSCN at mucosal surfaces, which is highly localized due to direct association of secreted LPO with the epithelial surface [22], and generates HOSCN gradients that repel bacteria away from the epithelial surface but are non-toxic to the underlying epithelium. The molecular mechanisms by which H_2O_2 or HOSCN promotes such negative chemotaxis are as yet unspecified, and likely involve activation of redox-sensitive transcriptional programs [23], but might also relate to direct oxidative modifications of exposed cysteines within bacterial proteins that control chemotaxis, such as CheA/CheZ (e.g. [24]). Regardless of the antimicrobial mechanism, it is apparent that H_2O_2 production by DUOX plays an important role in minimizing epithelial infection by harmful pathogens, which is particularly essential in conditions of epithelial injury or barrier disruption when infection risk is increased [21]. As such, these antimicrobial actions of DUOX-dependent H_2O_2 represent a crucial aspect of appropriate wound healing responses, and contribute to minimizing chronic infection or injury.

H_2O_2 AS A MEDIATOR: ROLE IN REDOX SIGNALING AND GENE REGULATION

The discovery of different NOX enzymes in diverse cell lineages has altered the general concept of H_2O_2 and other ROS as primarily toxic or damaging molecules, and has

increased appreciation for these reactive molecules as cellular second messengers in e.g. cytokine or growth factor signaling pathways through redox signaling mechanisms (e.g. [14]). This concept also applies to epithelial DUOX which, in addition to generating extracellular H₂O₂ as an antimicrobial mechanism, also participates in cellular signaling pathways that control the expression of various genes involved in inflammation and wound responses [25, 26]. For example, our original *in vitro* studies of airway epithelial wounding indicated that activation of DUOX1 contributes to the cellular activation of extracellular signal-regulated kinase (ERK1/2) and induction of matrix metalloproteinase 9 (MMP-9), a critical mediator of epithelial wound repair [8]. Other studies similarly implicated DUOX1 in activation of ERK1/2 and/or the transcription factor nuclear factor (NF)- κ B in transcriptional regulation of various wound genes in addition to MMP-9, such as vascular endothelial growth factor (VEGF), the neutrophil chemokine interleukin (IL)-8 (CXCL8), and mucin genes (e.g. MUC1 and MUC5AC) [14, 27]. The precise mechanisms by which DUOX induces these responses are not yet fully established, but they commonly involve activation of epidermal growth factor receptor (EGFR), initiated by H₂O₂-dependent production of EGFR ligands through stimulation of a *disintegrin and metalloproteinase* (ADAM) family sheddases such as ADAM17 [9, 27]. Although ADAM17 may be activated by direct oxidative mechanisms [9, 27], its activation also critically depends on the non-receptor tyrosine kinase Src [9], an important mediator of cell migration and epithelial wound healing [10, 28]. Moreover, it is becoming increasingly clear that the Src family kinases (SFK), which comprise 9 members including Yes, Fyn, and Lyn, are not only controlled by phosphorylation/dephosphorylation events [28], but also by redox regulation of several conserved cysteine residues in cysteine cluster (CC) motifs within their kinase domain [29, 30]. Indeed, a number of studies have demonstrated that H₂O₂-dependent activation of Src is associated with oxidation of several cysteine residues and that mutants lacking these cysteine are refractory to activation. Recent studies in zebrafish demonstrated the importance of the SFK Fynb in tail fin regeneration after injury, and the importance of DUOX for Fynb activation by an oxidative mechanism [6]. ATP-mediated wound responses in airway epithelial cells similarly involve oxidative activation of Src, which is mediated by DUOX1 [9]. The close association of DUOX with Fynb in wounded tail fins [6], and of DUOX1 and Src in ATP-stimulated airway epithelial cells [9], strongly suggests a direct oxidative activation mechanism of these SFK's by DUOX-generated H₂O₂. While these various findings strongly support a role for SFK's as proximal redox sensors in e.g. wound responses, the precise oxidative cysteine modification(s) or their direct consequence for SFK activity have not been established. Moreover, contrasting the identification of specific cysteines involved in SFK activation by ROS or by thiol-reactive heavy metals [29, 30] are findings that oxidative mechanisms can also inactivate Src, e.g. through homodimerization by disulfide linkage involving Cys277 [31]. These apparently conflicting findings would implicate that SFK may be variably regulated by both direct and indirect redox mechanisms, depending on the redox state of the cell or the degree of ROS production, and may in fact serve as molecular switches to dynamically control ROS-dependent wound responses.

In spite of these compelling findings linking DUOX activation with SFK-dependent signaling, it is feasible that DUOX-derived H₂O₂ may also target alternative redox-sensitive protein targets, such as EGFR itself (e.g. [32]) or other DUOX-interacting proteins such as

the EF-hand binding protein, EFP1, which contains two thioredoxin domains and is likely involved in redox signaling (reviewed in [14]). Furthermore, it is important to point out that other ROS sources (other NOX's, mitochondria) may also contribute to redox-dependent wound responses. Future studies with refined technical strategies to identify specific redox-modifications within target proteins, combined with selective approaches to inhibit specific NOX/DUOX isozymes, will help shed further light on this issue and more definitively establish the mechanisms by DUOX or other cellular sources of H₂O₂ participate in wound responses or in cellular redox signaling in general.

H₂O₂ AS A MESSENGER: INFLAMMATORY CELL RECRUITMENT

Tissue injury is closely intertwined with infection and immune responses. As such, recruitment of neutrophils, monocytes and macrophages to the wound site is a common and critical feature of wound healing, as these cells play important roles minimizing wound infection, controlling the promotion and resolution of inflammation, removal of apoptotic cells, and promoting anti-inflammatory and replicative processes [33, 34]. Because these inflammatory cell types are themselves major sources of ROS, they are likely to affect redox-dependent wound responses, and in chronic conditions are likely to promote oxidative stress and tissue injury. However, intriguing recent studies in zebrafish tail fin injury models have elegantly shown that early H₂O₂ production by injured epithelia (due to DUOX activation) is in fact responsible for attraction of neutrophils, as the first responders of innate immunity, into injured tissue [5, 35]. Because of the detection of H₂O₂ gradients reaching 100-200 μ m from the wound margin, and findings that H₂O₂ may be directly chemotactic, it was suggested that wound-associated H₂O₂ acts as a direct neutrophil chemoattractant to guide neutrophils toward the injury area [5]. Similar studies in wounded *Drosophila* embryos illustrated DUOX-derived H₂O₂ as a chemoattractant signal for hemocytes (*Drosophila* macrophages) into the wound area [36]. More recently, it was established that such oxidant-dependent neutrophil chemotaxis is related to specific oxidation of a cysteine residue (C466) in the neutrophil SFK, Lyn, thereby promoting its activation near the leading edge of migrating neutrophils [35]. The involvement of DUOX in neutrophil recruitment was recently also documented in a mouse model of allergic inflammation [37], as a potential wound response since allergic airway inflammation is commonly associated with airway epithelial injury or dysfunction.

Contrasting or complementing the postulated function of wound-derived H₂O₂ as a chemotactic signal for neutrophils or other leukocytes, injurious and microbial stimuli also promote the epithelial production of the neutrophil chemoattractant IL-8 (CXCL8), which is mediated by DUOX-dependent cell signaling pathways, as mentioned previously. The chemotactic properties of IL-8 involve CXCR-dependent activation of the neutrophil NADPH oxidase NOX2, which may contribute to their recruitment by localized redox signaling and oxidative activation of SFKs such as Lyn [38]. The importance of IL-8 homologs in wound-induced neutrophil recruitment was also recently established in zebrafish tail fin injury models, which involves the contribution of their two CXCL8 homologs Cxcl8-l1 and Cxcl8-l2, with variable effects on neutrophil recruitment and migration speed [39]. Since neutrophils are typically heterogeneous and may exist as distinct functional populations, and their chemotactic mechanisms differ depending on the activating

signal or context of injury or infection [40], their relative responses to Cxcl8 and/or H₂O₂ may be quite variable.

The preceding paragraphs illustrate our current understanding of the permissive role of early H₂O₂ production, as a proximal damage signal, in several aspects of the wound response. As illustrated in Fig. 2, these H₂O₂-mediated responses are initiated by common activation of DUOX (in e.g. zebrafish or *Drosophila*) and DUOX1 in the context of airway epithelial injury. Of course, these early wound response represent only a minor component of overall ROS-dependent mechanisms in tissue injury or inflammation, and likely also involve additional ROS sources in epithelial cells, and most certainly in recruited inflammatory-immune cells (due activation of their NADPH oxidase, NOX2). In fact, NOX2 plays diverse roles, not only in microbial killing, but also in neutrophil or macrophage inflammatory signaling, cytokine production, and survival [15, 33], and tight control of such ROS production or metabolism are critical in appropriate wound responses. For example, the presence of myeloperoxidase in neutrophils, critical in H₂O₂ metabolism by, was found to contribute importantly to H₂O₂ removal and termination of neutrophil recruitment in wound responses [41]. It follows that uncontrolled ROS production or defective ROS metabolism, are likely to result in inappropriate or defective wound responses and promote chronic injury and inflammation. At present, our understanding of the overall roles of ROS in various aspects of tissue injury and inflammation is still fairly limited, which is largely related to incomplete knowledge of the specific roles of specific diverse ROS sources, with either specific or complementary and redundant roles, and the precise molecular mechanisms by which ROS affect cell function.

EVALUATING H₂O₂-DEPENDENT SIGNALING: ANALYSIS OF REVERSIBLE CYSTEINE OXIDATION

The biological actions of H₂O₂ are governed by its chemical reactivity towards biological targets, which include metallo-enzymes such as heme peroxidases as well as oxidant-sensitive amino acid residues such as cysteine [14]. Cysteines are often highly conserved in proteins, especially in cysteine clusters, and frequently have structural and/or functional importance. Cysteine is also selectively used in proteins, and is among the least abundant amino acids with lower than expected occurrence based on codon usage, although it is used more prevalently in higher organisms [42]. Since the evolution of increasingly complex multicellular organisms is also associated with increased diversity of NOX enzyme systems (major sources of regulated ROS production) [43], such increased cysteine usage in proteins likely reflects their common role in functional protein regulation by oxidative mechanisms. Indeed, due to its strong nucleophilic properties, cysteine thiols (–SH) are highly reactive towards H₂O₂, especially in their deprotonated thiolate (–S[–]) form. Because the pK_a of protein cysteines varies widely, depending e.g. hydrogen bonding with nearby amino acid side chains or coordination with metal ions (Zn²⁺, Fe^{2+/3+}) to stabilize –S[–], reactions of H₂O₂ with protein cysteines kinetically range over seven orders of magnitude. Another level of diversity exists with respect to the nature of cysteine oxidation. Oxidation of cysteine thiols by H₂O₂ initially generates a sulfenic acid (–S–OH; Figure 3), which exhibits both electrophilic and nucleophilic properties and is therefore intrinsically reactive and typically

exist only transiently and is readily converted to more stable products. In conditions of oxidative stress, with high or persistent ROS production, sulfenic acids can be further oxidized to sulfinic and sulfonic acids ($-S-O_2H$ and $-S-O_3H$, respectively; Fig. 3). However, due to its strong nucleophilic behavior, sulfenic acids preferably react with other available cysteines under physiologic conditions, to form intra- or intermolecular disulfide bonds (with other proteins or the abundant thiol-containing small molecule, GSH; Figure 3), or with nitrogen nucleophiles to form a more stable sulfenamide [44]. On one hand, these secondary reactions may serve to protect sulfenic acids from further oxidation, but they also expand the spectrum of redox signaling modes with diverse structural or functional consequences. Since no direct evidence appears to exist to indicate that sulfenic acids act directly as signaling molecules, they most likely serve as intermediates in redox signaling through disulfide formation [45]. Depending on the nature of the protein cysteine involved, or its oxidative modification, functional consequences of cysteine oxidation may vary, and range from preventing the active role(s) of cysteine in catalytic enzyme mechanisms (e.g., protein tyrosine phosphatases) to induction of structural alterations or covalent protein-protein interactions, with more wide-ranging functional consequences. The functional importance of such oxidative cysteine modifications in redox-dependent cell signaling pathways is further exemplified by the fact that they are readily reversible by the actions of highly conserved oxido-reductases of the thioredoxin and glutaredoxin families that help restore the original cysteine redox states (Fig. 3).

Because of the diverse and often reversible nature of oxidative posttranslational protein cysteine modifications (which extends beyond H_2O_2 -dependent signaling since cysteines are also subject to other oxidative modifications such as *S*-nitrosylation, *S*-alkylation, and *S*-sulfhydration), our overall appreciation and understanding of the functional significance of these various cysteine modifications is still rather limited, although this research field is rapidly emerging [46]. In fact, the importance of cysteine oxidation or disulfide linking in protein regulation has long been underappreciated due to the common use of reducing agents in protein analyses, which reverse such cysteine modifications. Moreover, in spite of significant technical advances, reliable analysis of specific oxidative cysteine modifications and their biological consequences remains a major challenge. Involvement of cysteine oxidation/modification in the context of biological signaling or oxidative stress has most commonly been demonstrated using thiol-specific labeling approaches, to quantitatively determine loss of reduced cysteines in specific target proteins. Combined with strategies to e.g. inhibit NOX activation or using cysteine-lacking mutants, these approaches have been useful in identifying specific protein cysteine targets. Indeed, such approaches have revealed the importance of Src family kinases as proximal redox sensors in H_2O_2 -dependent wound responses [6, 9] or neutrophil recruitment [35]. However, such approaches cannot elucidate the nature of cysteine modifications or their functional repercussions, nor do they definitively establish whether these identified cysteines were directly targeted by H_2O_2 or were modified by more indirect mechanisms. It would therefore be desirable to be able to directly determine specific cysteine modifications, which is clearly much more challenging because of their highly variable nature. Indirect approaches to survey specific oxidative cysteine modifications have utilized (selective) reduction strategies prior to thiol-specific labeling, in e.g. biotin switch approaches (e.g. [46]), which have helped gain some further

insights although they suffer from lack of selectivity or specificity. For example, it is difficult to distinguish disulfide linking as a result of e.g. NOX-dependent redox signaling from disulfide linking during protein processing and maturation. An important advance has been the development of antibodies against specific protein cysteine modifications, such as *S*-glutathionylation or *S*-nitrosylation, as they allow more direct assessment of these specific modifications in target proteins. Complementary methods, such as use of biotin-tagged GSH, have also helped identify protein targets for *S*-glutathionylation, and these approaches have revealed specific and dynamic *S*-glutathionylation in a number of proteins with known roles in cytoskeletal dynamics and cell migration in various experimental settings, such as actin [47], low molecular weight protein tyrosine phosphatase (LMW-PTP) [48], the MAPK phosphatase MKP-1 [49], or the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [50], and it is feasible that such *S*-glutathionylation events contribute to epithelial wound repair or recruitment of inflammatory-immune cells during tissue injury, although its overall role appears to vary depending on cell type [50, 51].

Since *S*-glutathionylation can be initiated by various distinct mechanisms, not necessarily restricted to H_2O_2 (e.g. [52]), evaluation of H_2O_2 -specific redox signaling events ideally involves more direct analysis of the initial oxidative event in its signaling pathway, ie. sulfenic acids (-S-OH; Fig. 3). Efforts to trap intermediate formation of S-OH include reaction with the electrophilic reagent 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), which forms a sulfoxide adduct with an absorbance maximum distinct from its reaction products with cysteine [53]. More selective approaches to detect -S-OH have relied on its reactivity with nucleophiles such as dimedone (a 1,3-dione) or β -ketoesters to form a stable thioether, and a number of fluorescent and affinity-based probes have been developed based on functionalized derivatives of 1,3-cyclohexane or β -ketoesters, that have allowed for visualization or detection of -S-OH in target proteins [53-55]. In an attempt to improve cell permeability of dimedone-based probes to allow for trapping of intermediate -S-OH in intact cells prior to cell disruption, various azide- or alkyne-functionalized dimedone-based probes have been designed, with different cleavable linker regions, in an attempt to optimize -S-OH trapping and recovery for proteomic studies. These novel approaches have led to some exciting new insights into oxidative EGFR activation [56], and will be highly useful in future studies towards H_2O_2 -dependent signaling in e.g. wound responses or inflammation.

FINAL THOUGHTS

As outlined in this perspective, a growing body of recent evidence supports a critical role for H_2O_2 as an early damage signal in early epithelial or epidermal wound healing responses, which contrasts the common view of ROS as being mostly detrimental in conditions of tissue injury and inflammation. Studies in various organisms, and our own recent studies in mammalian systems, implicate the NADPH oxidase DUOX as the main source of H_2O_2 in epithelial wound responses, which include direct antimicrobial actions as well as involvement in redox signaling mechanisms that promote epithelial migration and leukocyte recruitment. In spite of these major advances, much remains to be learned regarding the relative involvement of DUOX or other sources of ROS (other NOX isozymes or NOX-independent mechanisms) and their dynamic roles initial wound responses, inflammatory signaling, or chronic tissue injury. The advent of novel experimental strategies to detect and

quantify protein cysteine oxidation, and continued development of genetic or pharmacological tools to evaluate the contributions of individual NOX enzymes, will shed further light on this and may help dissect the beneficial and potentially harmful actions of ROS in tissue injury and inflammation, especially in chronic diseases.

In spite of our rapidly advancing understanding of redox signaling, several fundamental concepts are still incompletely understood. For example, it is often considered that NOX-dependent redox signaling is highly localized and occurs in a proximity-based fashion in which redox-regulated targets are actively recruited into redox signaling complexes [9, 57], which appears to contrast with observations of DUOX-dependent H_2O_2 gradients over multiple cell diameters (e.g. [5, 35, 36]), which would imply more far-ranging signaling events. Since most cells express multiple NOX isoforms or produce ROS by alternative mechanisms, this would suggest that they may participate in complementary or redundant redox signaling events, that are primarily controlled at the level of localized NOX activation and ROS production rather than localization of their targets. Such more distant redox signaling events would also be highly subject to the actions of H_2O_2 -metabolizing enzymes such as GSH peroxidases, peroxiredoxins, etc. In fact, rather than serving to metabolize and detoxify H_2O_2 , some lines of evidence indicate that these enzyme systems may in fact themselves participate in propagating redox signals (e.g. [58]).

To address these various issues and establish the critical importance of specific redox modifications in biology, we need improved approaches that allow for both quantitative and dynamic assessment of protein cysteine modifications in intact cellular systems. In this regard, newly developed experimental approaches to detect $-S-OH$ as the most proximal product of H_2O_2 reaction with cysteine are likely to be most helpful in identifying direct targets for e.g. DUOX-derived H_2O_2 , although it should be realized that other biological oxidants, such as peroxynitrite or hypohalous acids, also oxidize $-SH$ to $-S-OH$. Therefore, detection of $-S-OH$ in specific proteins should always be accompanied by selective approaches to inhibit specific NOX/DUOX enzymes, to establish specific cause-effect relationships. Unfortunately, the current lack of specific available inhibitors for specific NOX/DUOX enzymes is an impediment to this research field. Also, a major challenge with respect to detection of $-S-OH$ remains that this intermediate is often short-lived and currently developed probes have either limited reactivity or chemical selectivity, thus leading to potential false positives or negatives [53]. Complementary approaches to detect formation of disulfides (such as protein S-glutathionylation) as more stable products may not only help overcome this limitation, but will also offer additional insight into the potential functional consequences of redox-dependent protein signaling, and their regulation by thioredoxin or glutaredoxin oxidoreductases.

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Figure 1. Interrelationship between Ca^{2+} , ATP and H_2O_2 as early damage signals after epithelial injury

Mechanical or chemical epithelial injury results in increased intracellular Ca^{2+} through e.g. connexin channels (Cx) or transient receptor potential (TRP) channels, and thereby promoting the secretion of ATP. Extracellular ATP can activate purinergic P2Y or P2X receptor subtypes on the cell surface to stimulate Ca^{2+} -dependent signaling and activation of PKC, and thereby stimulates H_2O_2 production e.g. by activating DUOX. H_2O_2 can diffuse into target cells to initiate redox signaling and can also promote Ca^{2+} influx as well as ATP efflux, further indicating the close interplay between these conserved damage signals.

Figure 2. Actions of DUOX-derived H_2O_2 in epithelial wound responses

1) DUOX-derived H_2O_2 at the luminal surface creates an H_2O_2 gradient that repels bacteria to minimize infection, and produces antimicrobial HOSCN by lactoperoxidase (LPO)-catalyzed oxidation of SCN^- . 2) DUOX-derived H_2O_2 participates in intracellular redox signaling, e.g. through EGFR/ERK activation, and thereby regulates cytoskeletal dynamics and promotes expression of wound genes such as MMP-9, to stimulate epithelial cell migration. 3) DUOX-derived H_2O_2 promotes recruitment of neutrophils as a critical component of the wound response, by direct chemotactic properties and/or by increased production of the neutrophil chemokine IL-8.

Figure 3. Mechanisms of H_2O_2 -dependent redox signaling through cysteine oxidation

H_2O_2 can oxidize select protein cysteine thiols (existing as thiolate anion due to low pK_a) to initially form sulfenic acid (RSOH), which in turn can react with either cellular GSH to form RSSG (mixed disulfide with GSH, also known as *S*-glutathionylation), neighboring protein cysteines to form intra- or intermolecular disulfides, or with $\text{H}_2\text{O}_2/\text{ROS}$ to form sulfinic (RSO_2H) and sulfonic (RSO_3H , not shown) acids. With the exception of $\text{RSO}_{2/3}\text{H}$, these various cysteine oxidations can be reversed by thioredoxin (Trx)/glutaredoxin (Grx) systems, thus constituting reversible H_2O_2 -dependent redox signaling. In select cases, RSO_2H can be reversed back to ROH by sulfiredoxins (Srx).