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Hydrogen Peroxide as a Damage Signal in Tissue Injury and Inflammation: Murderer, Mediator, or Messenger?

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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 protein dysfunction, genetic instability, 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. J. Cell. Biochem. 115: 427–435, 2014.

KEY WORDS: REACTIVE OXYGEN SPECIES; NOX/DUOX; REDOX SIGNALING; CELL MIGRATION; CHEMOTAXIS; CYSTEINE

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, for example, 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 [Schafer and Werner, 2008; Mittal et al., 2013]. 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 [Roy et al., 2006; Gauron et al., 2013]. Over the years, it has become popular belief 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

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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 [Niethammer et al., 2009; Yoo et al., 2012; Gauron et al., 2013; Love et al., 2013]. 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 [Roy et al., 2006], 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 [Wesley et al., 2007; Gorissen et al., 2013; Sham et al., 2013]. 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 to 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 [Yoo et al., 2012; Cordeiro and Jacinto, 2013]. 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, for example, 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 [Cordeiro and Jacinto, 2013]. 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 [Niethammer et al., 2009; Yoo et al., 2012; Razzell et al., 2013]. 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 [Cordeiro and Jacinto, 2013].

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

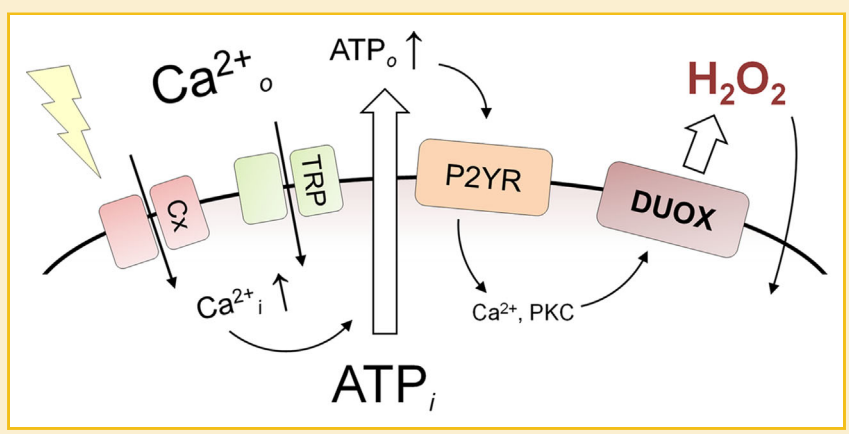

Fig. 1. Interrelationship between Ca^{2+}, ATP, and H_2O_2 as early damage signals after epithelial injury. Mechanical or chemical epithelial injury results in increased intracellular Ca^{2+} through, for example, connexin channels (Cx) or transient receptor potential (TRP) channels, and thereby promoting 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, for example, 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.

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 [van der Vliet and Bove, 2011]. 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 [van der Vliet and Bove, 2011], and conversely, Ca^{2+}-dependent signaling mechanisms can also contribute to promoting ATP release [Cordeiro and Jacinto, 2013], 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 [van der Vliet, 2008]. Recent studies in, for example, 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 [Yoo et al., 2012; Razzell et al., 2013]. 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 [van der Vliet, 2008]. 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 [Wesley et al., 2007; Sham et al., 2013]. Cellular production of H_2O_2 (or related ROS) appears to be a common result of concerted actions of Ca^{2+}-dependent signaling as well as ATP-dependent purinergic signaling, which promotes activation of, for example, protein kinases A or C that also contribute to NADPH oxidase activation [van der Vliet, 2008]. Because of the various biological actions of H_2O_2, which will be discussed in the following sections, cellular production of H_2O_2 can be viewed as a third early damage signal, that is closely associated with the other two damage signals, Ca^{2+} and ATP. This intricate association is further exemplified by findings that H_2O_2 production can itself contribute to Ca^{2+} influx as well as ATP release [van der Vliet, 2008], indicating that the precise relationships between these damage signals are complex and reciprocal.

It is apparent from the above that production of H_2O_2 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 H_2O_2 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 H_2O_2 exerts these actions is not always fully understood, as the biological actions of H_2O_2 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 H_2O_2-dependent redox signaling targets, and their application will be critical to establish the central mechanisms in H_2O_2-dependent injury responses as well as their regulation by other external factors.

H_2O_2 AS A MURDERER: ANTIMICROBIAL AND REPELLANT PROPERTIES

The deliberate production of H_2O_2 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 [Segal et al., 2012]. 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 H_2O_2 production as a potential host defense mechanism [van der Vliet, 2008]. 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 H_2O_2 to generate more potent antimicrobial oxidants, such as HOSCN [Moskwa et al., 2007; Gattas et al., 2009] (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 [Ha et al., 2005; Flores et al., 2010]. A critical aspect of this DUOX-dependent host defense mechanism includes the fact that pathogen-induced mechanisms also lead to induction of DUOX expression [Lee et al., 2013]. 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 [van der Vliet, 2008]. Intriguing recent studies also indicate that DUOX is selectively engaged in antimicrobrial 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 [Lee et al., 2013]. 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

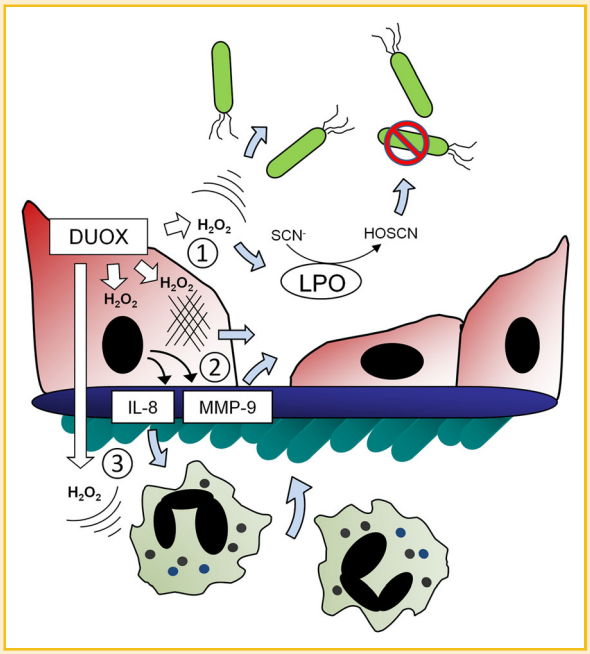

Fig. 2. Actions of DUOX-derived H_2O_2 in epithelial wound responses. (1) DUOX-derived H_2O_2 at the luminal surface creates an H_2O_2 gradient that repels bacteria to minimize infection, and produces antimicrobial HOSCN by lactoperoxidase (LPO)-catalyzed oxidation of SCN^-. (2) DUOX-derived H_2O_2 participates in intracellular redox signaling, for example, 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.

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 larger 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 [Allaoui et al., 2009]. 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 [Allaoui et al., 2009], and may also help clarify the more selective involvement of DUOX in preventing colonization by opportunistic pathogens without disturbing commensal mucosal bacteria [Lee et al., 2013]. 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 [Forteza et al., 2001], 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 [Dubbs and Mongkolsuk, 2012], but might also relate to direct oxidative modifications of exposed cysteines within bacterial proteins that control chemotaxis, such as CheA/CheZ [O'Connor and Matsumura, 2004]. 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 [Allaoui et al., 2009]. 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, for example, cytokine or growth factor signaling pathways through redox signaling mechanisms [van der Vliet, 2008]. This concept also applies to epithelial DUOX which, in addition to generating extracellular H_2O_2 as an antimicrobial mechanism, also participates in cellular signaling pathways that control the expression of various genes involved in inflammation and wound responses [Lee, 2009; Juarez et al., 2011]. 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 [Wesley et al., 2007]. Other studies similarly implicated DUOX1 in activation of ERK1/2 and/or the transcription factor nuclear factor (NF)-κB 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) [Koff et al., 2008; van der Vliet, 2008]. 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 H_2O_2-dependent production of EGFR ligands through stimulation of *a disintegrin and metalloproteinase* (ADAM) family sheddases such as ADAM17 [Koff et al., 2008; Sham et al., 2013]. Although ADAM17 may be activated by direct oxidative mechanisms [Koff et al., 2008; Sham et al., 2013], its activation also critically depends on the non-receptor tyrosine kinase Src [Sham et al., 2013], an important mediator of cell migration and epithelial wound healing [Boateng and Huttenlocher, 2012; Gorissen et al., 2013]. Moreover, it is becoming increasingly clear that the Src family kinases (SFK), which comprise nine members including Yes, Fyn, and Lyn, are not only controlled by phosphorylation/dephosphorylation events [Boateng and Huttenlocher, 2012], but also by redox regulation of several conserved cysteine residues in cysteine cluster (CC) motifs within their kinase domain [Senga et al., 2008; Giannoni et al., 2010]. Indeed,

a number of studies have demonstrated that H_2O_2-dependent activation of Src is associated with oxidation of several cysteine residues and that mutants lacking these cysteines 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 a oxidative mechanism [Yoo et al., 2012]. ATP-mediated wound responses in airway epithelial cells similarly involve oxidative activation of Src, which is mediated by DUOX1 [Sham et al., 2013]. The close association of DUOX with Fynb in wounded tail fins [Yoo et al., 2012], and of DUOX1 and Src in ATP-stimulated airway epithelial cells [Sham et al., 2013], strongly suggests a direct oxidative activation mechanism of these SFK′s by DUOX-generated H_2O_2. While these various findings strongly support a role for SFK′s as proximal redox sensors in, for example, 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 [Senga et al., 2008; Giannoni et al., 2010] are findings that oxidative mechanisms can also inactivate Src, for example, through homodimerization by disulfide linkage involving Cys277 [Sun and Kemble, 2009]. 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 H_2O_2 may also target alternative redox-sensitive protein targets, such as EGFR itself [Truong and Carroll, 2012] 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 van der Vliet [2008]). 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 H_2O_2 participate in wound responses or in cellular redox signaling in general.

H_2O_2 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 [Nathan, 2006; Koh and DiPietro, 2011]. 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 H_2O_2 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 [Niethammer et al., 2009; Yoo et al., 2011]. Because of the detection of H_2O_2 gradients reaching 100–200 μm from the wound margin, and findings that H_2O_2 may be directly chemotactic, it was suggested that wound-associated H_2O_2 acts as a direct neutrophil chemoattractant to guide neutrophils toward the injury area [Niethammer et al., 2009]. Similar studies in wounded Drosophila embryos illustrated DUOX-derived H_2O_2 as a chemoattractant signal for hemicytes (Drosophila macrophages) into the wound area [Moreira et al., 2010]. 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 [Yoo et al., 2011]. The involvement of DUOX in neutrophil recruitment was recently also documented in a mouse model of allergic inflammation [Chang et al., 2013], 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 H_2O_2 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 in turn contribute to their recruitment by localized redox signaling and oxidative activation of SFKs such as Lyn [Fialkow et al., 2007]. 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 [de Oliveira et al., 2013]. 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 [Deng et al., 2012], their relative responses to Cxcl8 and/or H_2O_2 may also be quite variable.

The preceding paragraphs illustrate our current understanding of the permissive role of early H_2O_2 production, as a proximal damage signal, in several aspects of the wound response. As illustrated in Figure 2, these H_2O_2-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 [Nathan, 2006; Segal et al., 2012], 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 H_2O_2 metabolism was found to contribute importantly to H_2O_2 removal and termination of neutrophil recruitment in wound responses [Pase et al., 2012]. 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 H_2O_2-DEPENDENT SIGNALING: ANALYSIS OF REVERSIBLE CYSTEINE OXIDATION

The biological actions of H_2O_2 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 [van der Vliet, 2008]. 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 [Jones and Go, 2011]. Since the evolution of increasingly complex multicellular organisms is also associated with increased diversity of NOX enzyme systems (major sources of regulated ROS production) [Kawahara et al., 2007], such increased cysteine usage in proteins likely reflects their common role in functional protein regulation by oxidative mechanisms. Indeed, due to their strong nucleophilic properties, cysteine thiols (−SH) are highly reactive towards H_2O_2, especially in their deprotonated thiolate ($-S^-$) form. Because the pK_a of protein cysteines varies widely, depending on hydrogen bonding with nearby amino acid side chains or coordination with metal ions (Zn^{2+}, $Fe^{2+/3+}$) to stabilize $-S^-$, reactions of H_2O_2 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 H_2O_2 initially generates a sulfenic acid (−S−OH; Fig. 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 sulfenic and sulfinic acids ($-S-O_2H$ and $-S-O_3H$, respectively; Fig. 3). However, due to their electrophilic characteristics, sulfenic acids readily 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; Fig. 3), or with nitrogen nucleophiles to form a more stable sulfenamide [Salmeen et al., 2003]. 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 [Roos and Messens, 2011]. Depending on the nature of the protein

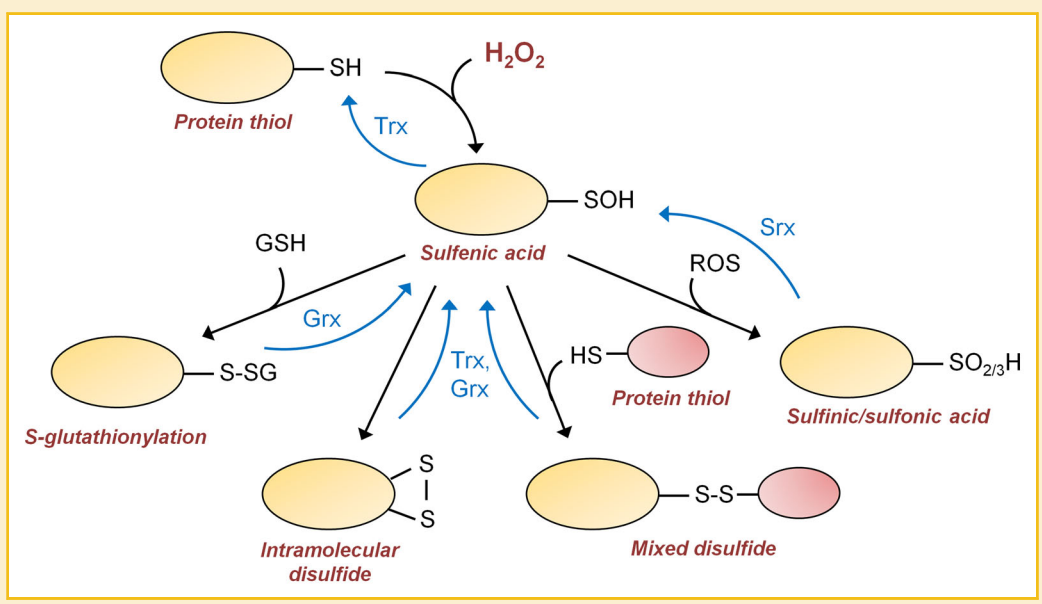

Fig. 3. Mechanisms of H_2O_2-dependent redox signaling through cysteine oxidation. H_2O_2 can oxidize select protein cysteine thiols (existing as thiolate anion due to low pK_a) to initially form sulfenic acid (RSOH), which in turn can react with either cellular GSH to form RSSG (mixed disulfide with GSH, also known as *S*-glutathionylation), neighboring protein cysteines to form intra- or intermolecular disulfides, or with H_2O_2/ROS to form sulfenic (RSO_2H) and sulfonic (RSO3H, not shown) acids. With the exception of $RSO_{2/3}H$, 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).

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 [Janssen-Heininger et al., 2008]. 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, for example, 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 [Yoo et al., 2012; Sham et al., 2013] or neutrophil recruitment [Yoo et al., 2011]. 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, for example, biotin switch approaches [Janssen-Heininger et al., 2008], 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, for example, 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 [Fiaschi et al., 2006], low molecular weight protein tyrosine phosphatase (LMW-PTP) [Abdelsaid and El-Remessy, 2012], the MAPK phosphatase MKP-1 [Kim et al., 2012], or the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [Evangelista et al., 2012], 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 [Evangelista et al., 2012; Sakai et al., 2012].

Since *S*-glutathionylation can be initiated by various distinct mechanisms, not necessarily restricted to H_2O_2 [Adachi et al., 2004], evaluation of H_2O_2-specific redox signaling events ideally involves more direct analysis of the initial oxidative event in its signaling pathway, that is, 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 [Gupta and Carroll, 2013]. 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 [Nelson et al., 2010; Qian et al., 2012; Gupta and Carroll, 2013]. 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 [Paulsen et al., 2012], and will be highly useful in future studies towards H_2O_2-dependent signaling in, for example, 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 [Ushio-Fukai, 2009; Sham et al., 2013], which appears to contrast with observations of DUOX-dependent H_2O_2 gradients over multiple cell diameters [Niethammer et al., 2009; Moreira et al., 2010; Yoo et al., 2011], 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 [Fomenko et al., 2011].

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 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 enxymes 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 [Gupta and Carroll, 2013]. 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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