Specificity in reactive oxidant signaling: think globally, act locally

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Although reactive oxidants have long been stigmatized as unwanted metabolic byproducts, the expression of oxidases specifically functioning to produce these same molecules in a regulated fashion is surprisingly pervasive throughout metazoan and plant evolution. Although the involvement of oxidants in many signaling pathways is well documented, the cellular strategies for conferring pathway specificity to such reactive molecules have remained more recondite. Recent studies now suggest that cells may spatially restrict oxidant production to allow microdomain-specific signaling.

Introduction

The now burgeoning field of oxidants in biological systems rose from obscurity with the cardinal discovery that the common red cell enzyme erythrocuprein was both ubiquitous and protective, functioning as a superoxide dismutase (SOD; McCord and Fridovich, 1969). The resultant syllogism therefore depicted free radicals as both ubiquitous and dangerous, and oxidant stress was rapidly established as a common mechanism linking inflammatory, degenerative, and neoplastic processes in human disease. The propensity of oxidants to initiate chain reactions and select targets based on redox potential rather than cellular function further suggested a capriciousness of oxidative reactions that destroyed the delicate biochemical specificity required by various signaling machines. Only in the past decade has it become clear that most, if not all, multicellular organisms have evolved molecular strategies to intentionally produce these unruly chemicals for, of all things, signaling purposes, prompting the question "whence specificity?"

The NADPH oxidases are evolutionarily ancient

Although they are not the only source of oxidants, the NADPH oxidase (Nox) family members are the principal complexes that function solely to redox-couple NADPH and molecular oxygen to generate $O_2^{\cdot -}$ and, thence, H_2O_2. Thus, the examination of Nox biology reveals much about the cellular logic behind regulated oxidant production. The seven known human Noxs include Nox1–5 and Duox1–2, with Nox2 (gp91^{phox}) being the founding member. As a family, these oxidases participate in a variety of adaptive functions, ranging from mitogenesis to immune cell signaling (Geiszt and Leto, 2004; Lambeth, 2004). Reflecting these varied biological roles, the Nox proteins have been implicated in several cell-fate pathways, such as the Ras mitogenic pathway (Irani et al., 1997), the MAP kinases (Gu et al., 2002; Xu et al., 2002), the JAK–STAT pathways (Schieffer et al., 2000), and NF-κB (Sulciner et al., 1996; Gu et al., 2003).

Nox-dependent signaling has been a biologically successful device by all accounts, having appeared early and persisted throughout evolution on the aerobic earth. Orthologous Nox genes arose in concert with multicellular organization (Lalucque and Silar, 2003), and so are found as early as the slime mold *Dictyostelium discoideum* and the filamentous fungus *Podospora anserina* (Malagnac et al., 2004; Lardy et al., 2005). During starvation conditions, free Dictyostelial amoebae aggregate into a slug that behaves as a single organism, differentiating a distinct organ, the spore-bearing fruiting body. Although single deletions of any of the three *nox* genes or *p22^{phox}* fail to produce a phenotype in unicellular amoebae, starvation of these knockout mutants interrupts fruiting body morphogenesis (Lardy et al., 2005). Similarly, deletion of either of the two *P. anserina* Nox genes results in failed fruiting body differentiation. (Malagnac et al., 2004). Thus, Noxs control developmental signaling in the most primitive multicellular organisms, an ancestral function that foreshadowed their later involvement in basic mammalian cell fate pathways. One might fairly ask why the utilization of reactive oxidants has been so evolutionarily durable and how oxidants can manage to selectively relay a diverse array of signaling cassettes, especially because the different Noxs presumably produce the same oxidant species perceived by the cell as an oxidative threat.

Physical organization of signaling elements is a common strategy for pathway specificity

A general paradigm in cell signaling holds that information proceeds through pathway-specific multimolecular complexes built

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Abbreviations used in this paper: AMPK, AMP-activated protein kinase; BCR, B lymphocyte antigen receptor; GEF, guanine nucleotide exchange factor; PTP, protein tyrosine phosphatase; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

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on colocalizing scaffolds as a means of maximizing efficiency and attaining specificity beyond what would be allowed by using the limited number of signaling proteins as individual, freely diffusible agents within a crowded cytosol. The logic behind such quaternary spatial organization would fit well with the use of oxidants as locally active mediators if two conditions are met. First, the source of oxidants should likewise be tightly regulated, not only from an agonistic standpoint but also in terms of strict subcellular localization. Second, a broad field of antioxidant activity must be present within the cytosol to confine oxidative effects to within proximity of their origin, in essence, optimizing spatial signal-to-noise ratios. The latter criterion has long been established, as several antioxidant enzymes are, in fact, largely cytosolic, such as Cu/Zn SOD and glutathione peroxidase. Pathways that produce oxidants as significant metabolic byproducts tend to be sequestered within organelles, whose defenses are correspondingly buttressed by higher concentrations of these or other antioxidant enzymes (such as catalase in peroxisomes or MnSOD in mitochondria). Even the exceptions to antioxidant distribution tend to prove the rule. For instance, peroxiredoxin II, through its association with PDGFR, suppresses PDGF signaling, whereas the less targeted catalase and glutathione peroxidase have no effect (Choi et al., 2005). What evidence exists that the former criterion, i.e., that oxidases are focally activated, is also fulfilled?

Nox subunits are directed to specific platforms

The cytoskeleton. Different cells, when imaged with different oxidant-detection methods, display subcellular restriction of oxidant activity around regions of cytoskeletal rearrangement (Fig. 1). Again, considering the Nox proteins as archetypal signaling oxidases, molecular links between the oxidase and the cytoskeleton have been described. Activation of the phagocyte oxidase, for instance, causes translocation of the adaptor $p47^{phox}$ and the activator $p67^{phox}$ to the cytoskeletal fraction such that the functioning oxidase is quantitatively cytoskeleton bound (Nauseef et al., 1991; El Benna et al., 1994). More recent studies have demonstrated constitutive cytoskeletal targeting of oxidase subunits in nonprofessional phagocytes such as endothelial cells (Gu et al., 2002; Li and Shah, 2002). In these cells, cytoskeletal disruption interrupts oxidant-mediated JNK signaling, suggesting a connection between cytoskeletal targeting, oxidant production, and the relay of signaling information (Gu et al., 2002).

A specific example of oxidant-dependent cytoskeletal function is the dependence of endothelial cell migration on Nox proteins (Moldovan et al., 2000; Ushio-Fukai et al., 2002). In these cells, oxidants concentrate within membrane ruffles, mirroring the distribution seen in stimulated adherent neutrophils (Fig. 1, A–C; Heyworth et al., 1997). Interestingly, $p47^{phox}$, which is the principal kinase target during oxidase activation, directly binds two proteins enriched within leading edge lamellipodia–moesin and WAVE1 (Wientjes et al., 2001; Wu et al., 2003). The latter protein catalyzes dendritic actin nucleation responsible for lamellar structure in a Rac1-dependent fashion; accordingly, $p47^{phox}$–WAVE1 complexes contain Rac1 and the Rac1 effector PAK1, and antioxidants or truncations that disrupt $p47^{phox}$–WAVE1 interactions diminish ruffle formation (Wu et al., 2003). $p47^{phox}$ also associates with cortactin, which is a protein involved in lamellipodial persistence, although it is unclear whether the proteins associate directly or indirectly through larger cytoskeletal complexes (Touyz et al., 2005).

Another targeting device for $p47^{phox}$ within specific lamellar structures appears to be TRAF4, an orphan that, unlike its paralogues, has not been demonstrated to actively function in innate immune signaling. Knockout models suggest that mouse

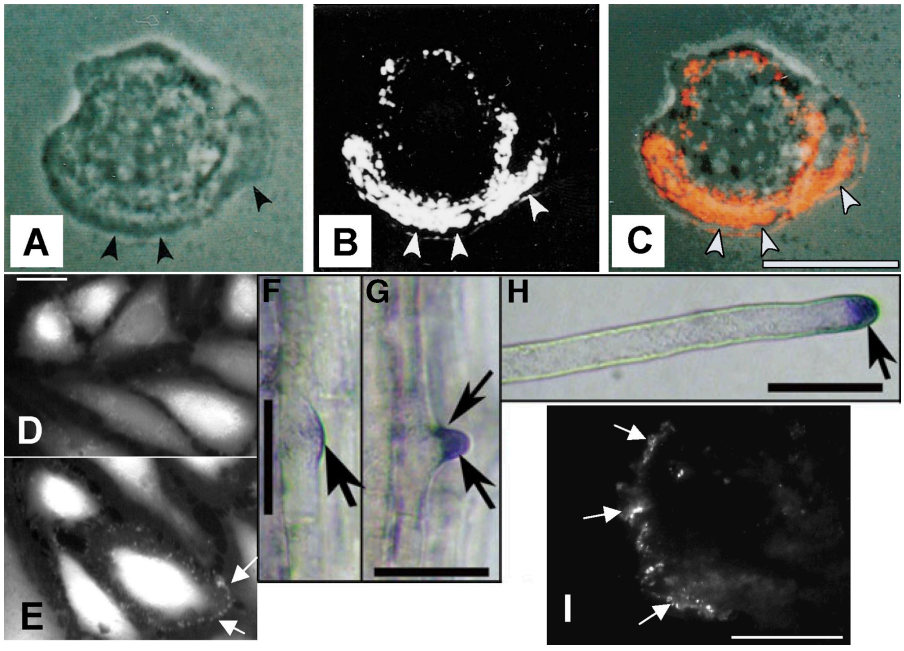

Figure 1. **Oxidant production is focal.** (A–C) An adherent human neutrophil was stimulated with PMA for 3 min, forming a broad lamellipodium (arrowheads). H_2O_2 was detected as a cerium perhydroxide reaction product (B) and was pseudocolored and overlaid on the phase-contrast image (C). Oxidants are heavily concentrated in the ruffling lamellipodium. (D and E) Endothelial cells were loaded with the oxidant-sensitive fluorescent dye dichlorofluorescein and imaged live. Cells expressed either an oxidase-inactive mutant $p47^{phox}$(W193R) (D) or a constitutively activating mutant $p47^{phox}$(S303,304,328D) (E). Activation of the oxidase is associated with focal oxidant production at cell edges (E, arrows). (F–H) Root hairs from the plant *A. thaliana* stained with nitroblue tetrazolium show oxidant production as the blue formazan reaction product. Oxidants are formed at the initiating bulge (F, arrows) and at the actively growing root tip (G and H, arrows). (I) An endothelial cell expressing a DsRed fusion of $p47^{phox}$ was imaged with total internal reflection fluorescence microscopy, showing discrete targeting of the oxidase protein to ventral leading edge structures, which were likely integrin complexes (arrows). A–C are reproduced with permission from Heyworth et al., 1997 in *Histochem. Cell Biol.*, Vol. 108. F–H are reproduced with permission from Macmillan Publishers Ltd. from Carol et al., 2005 in *Nature*, Vol. 438. Bars: (A–C and F–H) 10 μm; (D, E, and I) 20 μm.

TRAF4 and the *Drosophila melanogaster* orthologue dTRAF1 instead control ontogenic migration during respective dorsal closure events (Liu et al., 1999; Regnier et al., 2002). In the fly, dTRAF1 operates within a Rho-GTPase/JNK cassette during cell migration, and a parallel situation in human endothelial cells may require a direct interaction between TRAF4 and $p47^{phox}$. This interaction governs oxidant-dependent JNK activation, and endothelial cell migration involves TRAF4-dependent activation of the NADPH oxidase through the Rho-GTPases and PAK1 (Xu et al., 2002; Wu et al., 2005). TRAF4 and $p47^{phox}$ target focal integrin complexes within the lamellipodia of motile endothelial cells, tethered by the focal contact scaffold Hic-5. Thus, TRAF4 appears to function in this regard by focusing the activation of the oxidase to a specific cytoskeletal structure.

Besides $p47^{phox}$ phosphorylation, Rac1 activation is also required to activate many Noxs; thus, sites of Rac1 activation may also be expected to specify the subcellular location of Nox-dependent signaling complexes. Active Rac1, for instance, concentrates within ruffling lamellae, suggesting spatial coordination of Rac's cytoskeletal and prooxidant effects (Kraynov et al., 2000). One potential mechanism for Rac1 targeting is through the actin-binding scaffold IQGAP, which targets leading edge actin structures and mediates cell migration (Mataraza et al., 2003). IQGAP not only binds and, therefore, localizes the active forms of Rac1 and Cdc42 but also associates with VEGFR2 and Nox2 at leading edge structures, mediating VEGF-dependent oxidant production (Ikeda et al., 2005).

Another tactic cells use to spatially restrict Rac1 function is local exclusion of Rho-GDI. A striking example of how Rho-GDI specifies Nox activation sites was recently demonstrated in the plant *Arabidopsis thaliana*. Focal cytoskeletal rearrangements within the specialized trichoblast cell cause a single root hair to extend from each cell. A mutation resulting in the root hair–defective phenotype localizes to the gene for a plant Nox, RHD2/AtrbohC (Foreman et al., 2003). Although wild-type plants produced oxidants confined to the tip of extending root hairs (Fig. 1, F–H), *rhd2* mutants neither produced oxidants nor formed root hairs. Conversely, diffuse exposure to exogenous oxidants caused loss of spatial control, with the resultant formation of numerous aberrant root hairs. Two subsequent *A. thaliana* mutants causing a similar phenotype of multiple aborted growth bulges (*supercentipede*) were found to encode SCN1, which is a Rho-GDI (Carol et al., 2005). Whereas wild-type plants demonstrated a single focus of oxidant production at the growing root hair tip, *scn1* mutants displayed multiple foci of oxidants corresponding to abnormal growth bulges. Therefore, the plant Rho-GDI SCN1 functions to restrict oxidant production exclusively to a single root tip.

A third method of localizing Rac1 activation is through targeting of Rho guanine nucleotide exchange factors (GEFs) with Rac1 activity. Recruitment of a Rac1 GEF is suggested by the association of human Nox1 with the Rac GEF βPIX (Park et al., 2004). Thus, βPIX, which is known to modulate EGFR function, activates Rac1, causing EGF-dependent oxidant production. In addition, Rap1a, which associates with the Nox2 complex, targets membrane protrusions and locally activates the Rac GEFs Vav2 and Tiam1, and thus Rac1 itself, at the lamellipodial edge (Arthur et al., 2004).

Membrane rafts. Membrane rafts are known to facilitate the congregation of several signaling proteins, including Nox subunits. In suspended myeloid cells, for example, the Nox2 cytochrome subunits constitutively sequester in raft fractions, with translocation of the soluble proteins $p47^{phox}$ and $p67^{phox}$ into rafts after stimulation (Vilhardt and Van Deurs, 2004). Raft association of the mitogenic Nox1 has also been noted in smooth muscle cells (Hilenski et al., 2004), and angiotensin II stimulation, which proceeds through Nox1, promotes Rac1 trafficking into rafts, whereas raft disruption blocks angiotensin II–dependent oxidative signaling (Zuo et al., 2004). Similarly, rafts contain the focal complex–associated TRAF4, and raft disruption blocks TRAF4-dependent oxidative signaling (Wu et al., 2005).

The association of Nox proteins with raft microdomains may explain, in part, why oxidant production by Noxs, which is presumed to be directed outside the cell, can affect intracellular targets. Plasma membrane rafts containing Rac1 are known to be internalized in response to integrin signals (del Pozo et al., 2004), and caveola-derived signaling endosomes, which are a type of membrane-derived "signalosome," continue to transduce growth factor receptor signals after internalization. Indeed, small caveolin-containing vesicles termed cavicles are thought to be transported, possibly as microtubular cargo, between the plasma membrane and pericentrosomal caveosomes (Mundy et al., 2002). Although it is as yet unclear whether functioning Nox complexes are transported within similar internalized structures, Nox2, $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$ clearly exist in discrete, detergent-insoluble complexes within the cytosol of endothelial cells in association with microtubules (Gu et al., 2002; Li and Shah, 2002). More recently, IL-1β has been shown to activate Nox2 within early endosomes containing IL-1R (Li et al., 2006). The possible functioning of Nox complexes within these or other intracellular membranous structures warrants further investigation.

Mitochondrial oxidants and mitochondrial signaling

Mitochondria have long been known to represent focal sources of reactive oxidants, and more recently, have been appreciated as important signaling organelles. Mitochondria, for instance, regulate several facets of cellular energetics beyond ATP production, at least some through local oxidant production. AMP-activated protein kinase (AMPK), which is believed to serve as an energy gauge, is activated by mitochondrial oxidants, perhaps through mitochondrial c-Src (Zou et al., 2004). AMPK controls several energy-related pathways, including the inhibition of acetyl CoA carboxylase with suppression of fatty acid synthesis and the activation of glycolysis and β-oxidation. Under hypoxic conditions, mitochondrially derived oxidants cause activation of AMPK; the compound metformin, which is commonly used to treat diabetes, activates AMPK, again, through mitochondrial oxidant production (Zou et al., 2004; Quintero et al., 2006). Another mediator of cellular energetics is pyruvate, a watershed metabolite that drives mitochondrial respiration. Pyruvate-induced mitochondrial oxidants appear to activate JNK, leading

to inhibition of GSK-3β and activation of glycogen synthase, thus, sequestering glucose and lowering pyruvate in a negative feedback cycle (Nemoto et al., 2000). It is not clear whether this oxidative signaling is restricted to the local mitochondrial environment or what the proximal oxidant target is; however, both JNK and JNK scaffolds, as well as the putative downstream target GSK-3β, associate with mitochondria, allowing the possibility of a locally confined circuit (Wiltshire et al., 2002; Putcha et al., 2003). Mitochondrial redox signaling in response to nutrient availability is likely to have ancient roots. The simple colonial hydroid *Podocoryna carnea*, for instance, responds to changes in its food supply by adopting either dense feeding or runner-like searching colony morphologies. Interestingly, these morphologic changes appear to be controlled by changes in mitochondrial redox states (Blackstone, 2003). More generally, across many phyla several connections between cellular energetics, mitochondrial oxidants, and aging phenotypes have been noted (Balaban et al., 2005).

Perhaps at some level related to energy management, mitochondria also play a central role in programmed cell death; mitochondrial oxidants are well known to mediate this form of death. Less clear are the exact mechanisms by which mitochondria are stimulated to produce increased oxidants, and what the proximate targets of such oxidants are. Recently, the proapoptotic protein $p66^{Shc}$ was shown to localize to the mitochondrial intermembrane space and redox cycle with cytochrome *c* to produce oxidants that induce the permeability transition (Giorgio et al., 2005). Such oxidants are thought to locally target the inner mitochondrial membrane, causing both depolarization and cytochrome *c* release (Zamzami et al., 1995), although other targets may be important. $O_2^{\cdot -}$ produced outside of purified mitochondria, for instance, causes massive cytochrome *c* release without inner membrane damage in a process targeting the outer membrane voltage-dependent anion channel (VDAC; Madesh and Hajnoczky, 2001). It is unclear whether VDAC itself is an oxidant target or, perhaps more likely, is required for ingress of $O_2^{\cdot -}$ into the intermembrane space, but these data nevertheless support the notion that cytochrome *c* release proceeds as a result of local effects of mitochondrial oxidants.

How nature uses microdomains to integrate oxidants into signaling logic

One might anticipate, given the pervasion of reactive oxidants in a broad array of signal pathways, that rather than targeting many specific but thematically unrelated targets, oxidants may instead serve to modulate but a few signaling devices that are nonetheless widely used. Two such broad-use devices modulated by oxidants appear to be protein tyrosine phosphorylation cascades and intracellular Ca^{2+} transients, both of which are site specific in their effects. Although not the only cellular oxidant targets, these two systems serve to link oxidants to several pathways (Fig. 2).

Tyrosine phosphorylation. Interestingly enough, tyrosine phosphorylation is extensively used only by multicellular eukaryotes, to control cell fate decisions and cytoskeletal dynamics (Alonso et al., 2004). Thus, the appearance of Nox genes seems to have roughly coincided with the evolution of tyrosine phosphorylation–dependent signaling, both systems controlling broadly overlapping cellular functions. In addition, both tyrosine kinases and protein tyrosine phosphatases (PTPs) exert signal specificity through subcellular targeting capability, as well as catalytic domain specificity. PTPs in particular are sensitive to oxidative inactivation, owing to their invariant catalytic cysteine that, when held at a low pK_a by vicinal basic residues, becomes sensitive to oxidative attack by forming a thiolate anion. Within a local shell of oxidative influence created by targeted oxidants, selective oxidation of such nucleophilic-active site moieties provides some indication that not all local cysteines are equally susceptible to oxidative modification. An increasing number of PTPs have been shown to be regulated by oxidative modification during the course of physiologic signaling, such as LMW-PTP and SHP-2 by PDGF, PTP1B by EGF and PDGF, and MKP by TNF (Lee et al., 1998; Chiarugi et al., 2001; Meng et al., 2002; Kamata et al., 2005).

As subcellular location specifies both oxidase and PTP function, one might expect to find frequent colocalization of these systems. Ligation of the B lymphocyte antigen receptor (BCR), for instance, initiates tyrosine phosphorylation of receptor-associated proteins such as Lyn in a manner negatively

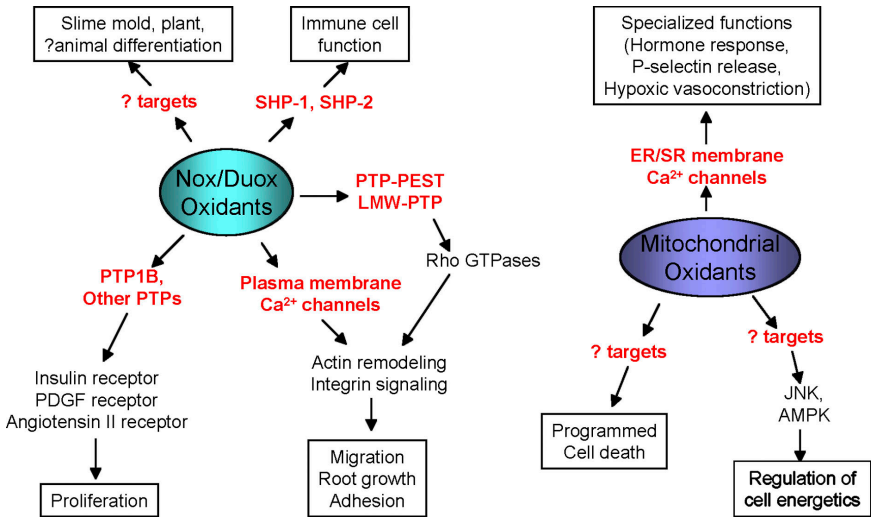

Figure 2. **Common oxidant sources signal through a variety of pathways.** Schematic indicates proposed oxidant targets in red, which dictate the pathways affected. Colocalization of the oxidant source and proximate target may provide pathway specificity. In several pathways, both proximate targets and intermediate steps are unclear.

regulated by the BCR-associated PTP SHP-1. Recently, BCR signaling was found to depend on oxidants produced by the Nox member Duox1 through oxidative inactivation of SHP-1 (Singh et al., 2005). Notably, only BCR-associated SHP-1, and not SHP-1 isolated from BCR-depleted cytosol, sustained oxidative inactivation, confirming the local effect of oxidants. In a similar fashion, T cell receptor cross-linking also induces an oxidant burst that is necessary for downstream integrin activation. This oxidant burst selectively inactivates SHP-2, which is recruited directly to the T cell receptor adaptor complex, but has no effect on SHP-1 (Kwon et al., 2005). SHP-2 is also oxidatively inactivated by PDGF stimulation in a process that requires its association with PDGFR, again indicating the importance of spatial proximity between oxidant source and PTP target (Meng et al., 2002).

In the context of cell migration, the phosphatase PTP-PEST has been found to target peripheral focal integrin complexes and thereby control lamellipodial dynamics. PTP-PEST binds Hic-5 and diminishes Pyk2 and Src function; therefore, TRAF4, by tethering $p47^{phox}$ to the Hic-5 complex, mediates oxidative modification of PTP-PEST, but not uninvolved phosphatases such as MKP and SHP-2, suggesting spatial restriction of PTP inactivation (Wu et al., 2005). Rac1-induced lamellipodial ruffling also requires oxidative inactivation of LMW-PTP (Nimnual et al., 2003). This inactivation results in phosphorylation (activation) of p190Rho-GAP, linking oxidant production with RhoA inactivation within leading edge ruffles. A similar mechanism may facilitate tyrosine phosphorylation of other integrin structures. Nox4 for instance, whose activity is independent of $p47^{phox}$, concentrates within focal adhesions in smooth muscle cells (Hilenski et al., 2004). Transfected Nox4 also colocalizes with PTP1B in COS7 cells, and Nox4 enhances insulin receptor tyrosine phosphorylation through PTP1B inactivation, which is again consistent with spatial coordination between oxidant source and target (Mahadev et al., 2004; Martyn et al., 2006).

Tyrosine phosphorylation events within mitochondria control energetics and cell death, much like mitochondrial oxidants. For instance, inactivation of Tim50, which is a mitochondrial inner membrane phosphatase, has been implicated in apoptotic cytochrome *c* release (Guo et al., 2004). Another phosphatase restricted to the mitochondrial inner membrane, PTPMT1, controls ATP production and insulin secretion (Pagliarini et al., 2005). Given that these PTPs are located in close proximity to the mitochondrial respiratory chain, local redox regulation would seem likely, although this specific relationship has not been well studied.

Intracellular Ca^{2+}. A striking similarity exists between the proposed compartmentalization of oxidant signaling and the spatial restriction of Ca^{2+} transients. Because of the limited diffusion of free cytosolic Ca^{2+}, both entry across the plasma membrane and release from sarcolemmal stores can result in focal Ca^{2+} accumulations such as puffs in *Xenopus laevis* oocytes, sparks in cardiac myocytes, and quantum emission domains in giant squid synapses. In the latter organ, Ca^{2+} concentrations of 300 μM confined within 0.5-μm regions have been reported. This spatial control of Ca^{2+} transients to specific microdomains is thought to be critical to the maintenance of Ca^{2+} signal fidelity. In vascular smooth muscle, for example, focal Ca^{2+} sparks cause relaxation, whereas diffuse increases in intracellular Ca^{2+} result in contraction. It should be noted that Ca^{2+} signals relay proliferative, cytoskeletal, and death signals, similar to focal oxidants. Is there coordination between such Ca^{2+}-dependent signaling and oxidant production?

Many Nox proteins, including human Nox5 and DUOX1/2, respond directly to Ca^{2+} through N-terminal EF-hand motifs, providing one mechanism by which Ca^{2+} increases local oxidant production. In plants, whose Nox proteins commonly possess EF-hand domains, calmodulin signals have also been shown to accentuate the Ca^{2+}-dependent oxidant burst (Harding et al., 1997). Even Nox forms that lack EF-hand domains have been shown to respond to Ca^{2+}. The response of Nox2 to Ca^{2+}, which is required for maximal activation, is mediated by two small proteins, MRP8 and MRP14, which are just large enough to contain two EF-hands. In response to Ca^{2+} transients, these proteins heterodimerize with each other, associate with Nox2, and enhance oxidase activation synergistically with $p47^{phox}$ and $p67^{phox}$ (Berthier et al., 2003). Possibly, therefore, some Nox proteins have retained their Ca^{2+}-response elements in trans rather than cis.

Conversely, oxidants also trigger focal Ca^{2+} signals, in part through direct activation of Ca^{2+} channels. Again in the plant *A. thaliana*, root growth requires a high local concentration of cytosolic free Ca^{2+} that starts at the initiating bulge and remains confined to the growing root tip, corresponding to the localization of oxidants. Mutants defective in the plant Nox RHD2 fail to establish Ca^{2+} gradients and, thus, lack normal root hairs, whereas diffuse application of exogenous oxidants, which create multiple aberrant root bulges, reactivates Ca^{2+} channels with resultant delocalized Ca^{2+} influx (Foreman et al., 2003). In endothelial cells, H_2O_2 decreases the threshold of inositol 1,4,5 trisphosphate required to release intracellular Ca^{2+} stores, revealing an alternative mechanism for influencing Ca^{2+}-dependent signaling (Hu et al., 2000). The capacity of intracellular Ca^{2+} and reactive oxidants to positively modulate each other, in concept, allows the rapid establishment of localized, positive feedback loops. After lymphocyte BCR ligation, for instance, downstream Lyn phosphorylation is dependent on both intracellular Ca^{2+} and Nox-dependent oxidant signaling, which positively modulate each other in a monostable on–off circuit (Singh et al., 2005).

Similar positive feedback loops also allow rapid asymmetric amplification of signals and early assignment of polarity to cellular processes such as directed chemotactic migration. At the leading edge of lamellar structures, these autoamplifying loops involve local activation of Cdc42, PAK1, and Rac1 (DerMardirossian et al., 2004), and also appear to involve an NADPH oxidase controlling focal complex dynamics (Wu et al., 2005). Not surprisingly, then, Ca^{2+} transients restricted to 2–3-μm foci within neuronal growth cones or fibroblast pseudopodial extensions have recently been observed, which are termed localized lamellipodial transients or localized fibroblast transients (Conklin et al., 2005). Both such Ca^{2+} transients increase with integrin activation and control tyrosine

phosphorylation events and focal complex turnover, much like focal oxidant production.

The spatial coordination of Ca^{2+} and oxidant signals is well-demonstrated in mitochondrial signaling. A principal intracellular store of Ca^{2+}, the ER, is arranged in intimate association with mitochondria in HeLa and mast cells (Rizzuto et al., 1998; Csordas et al., 1999). Opening of ER Ca^{2+} channels therefore results in local perimitochondrial Ca^{2+} levels >20-fold higher than elsewhere within the cytosol, causing mitochondrial Ca^{2+} uptake. Such coupled spikes in mitochondrial Ca^{2+} levels are thought to be important in propagating Ca^{2+} oscillations, activating mitochondrial enzymes, and possibly activating the permeability transition pore, and the physical proximity between the ER and mitochondrion has been likened to a privileged synaptic space within the cell (Csordas et al., 1999). Notably, ER Ca^{2+} channels associated with IP_3 and ryanodine receptors are oxidant sensitive; thus, adjacent mitochondrial oxidant production is necessary for physiologic Ca^{2+} oscillations after hormonal stimulation (Camello-Almaraz et al., 2006). This coupling between mitochondrial oxidant production and local Ca^{2+} transients also appears to be necessary for other specialized functions, such as endothelial P-selectin exocytosis and hypoxic vascular smooth muscle contraction (Ichimura et al., 2003; Michelakis et al., 2004). Interestingly, Ca^{2+} signals may link separate oxidative pathways. The marine plant *Fucus serratus*, for instance, responds to hyperosmotic stress with a transient polarized oxidant burst into the extracellular space at the rhizoid apex, which diffuses back into the cell tip to initiate an organized Ca^{2+} wave (Coelho et al., 2002). This Ca^{2+} signal is necessary for a subsequent oxidative signal originating from mitochondria, which then results in osmotic adaptation. Again, however, spatial restriction of oxidants is required, as diffuse application of H_2O_2 destroys the orderly Ca^{2+} wave propagation.

How do cells distinguish homeostatic signaling from oxidative stress?

To the extent that homeostatic signaling requires spatial confinement of oxidants, the cell may in many instances recognize oxidative stress through the detection of diffuse cytosolic oxidants that have escaped their usual designated locations. The appearance of oxidants out of an appropriate spatial context may represent reasonable cause to alert the cell to a dangerous excess of exogenous or pathologically controlled endogenous oxidants, to activate either defense or fail-safe death programs.

The model whereby subcellular localization of oxidants (or lack thereof) discriminates homeostatic from stress signaling allows several predictions. First, oxidative stress pathways should be triggered in response to the delocalized appearance of cytosolic oxidants. Typically, for instance, oxidative stress pathways are activated by suffusing the cell or organ with membrane-permeant oxidants such as H_2O_2 or by irradiation with UVB, both of which would be expected to blanket the cell with oxidants. Hypoxia reoxygenation also induces oxidative stress; it does so in many tissues via xanthine oxidase, which is a cytosolic protein. Mitochondria serve as a principal source of oxidants in several forms of oxidative stress, including reoxygenation and hyperoxia states. Both of the latter conditions increase global indices of cellular oxidative effects, indicating significant escape of oxidants into the cytoplasm. Indeed, mitochondrial release of $O_2^{\cdot -}$ into the cytosol is controlled by VDACs, and mice with heterozygous deficiency of mitochondrial SOD sustain oxidative damage to nuclear, as well as mitochondrial, DNA (Han et al., 2003; Van Remmen et al., 2003). Even in yeast, senescence accompanies the accumulation of oxidatively modified proteins, more than half of which are cytosolic (with the remainder being mitochondrial; Aguilaniu et al., 2003).

Second, one would expect to find oxidative stress reporters free within the cytosol. The early prototypes OxyR and Yap1p are found within the bacterial protoplasm and yeast cytosol, respectively, and become activated in response to exogenous H_2O_2 through disulfide bond formation and transcriptional activation (Zheng et al., 1998; Gulshan et al., 2005). Redox-sensitive cytosolic reporters that translocate into the nucleus persist in mammals. Redox factor-1, for instance, translocates from a diffuse cytosolic location into the nucleus with oxidative stress to facilitate the DNA-binding activity of NF-κB (Angkeow et al., 2002). Thioredoxin also functions as an oxidative stress reporter, moving into the nucleus to activate NF-κB, AP-1, and p53 (Hirota et al., 1999; Ueno et al., 1999).

Third, the cell may be expected to deploy the oxidative stress mechanism in response to other forms of cellular stress through a secondary increase in cytosolic oxidants. Heat shock, for instance, increases mitochondrial oxidant production, thereby activating HSF-1 through oxidant intermediates, whereas antioxidants diminish the heat shock response in *Saccharomyces cerevisiae* (Huang et al., 1994; Davidson and Schiestl, 2001; Ahn and Thiele, 2003; Moraitis and Curran, 2004). Heavy metals induce a large burst of H_2O_2 that activates the cytosolic factor HSF-1 (Ozaki et al., 2000). Notably, this oxidant production is suppressed by Rac1(N17), suggesting specific oxidant regulation. Finally, p53 not only responds to oxidative stress within the cytosol but also activates stress pathways by increasing mitochondrial oxidant production. The induction of oxidative stress simply through p53 overexpression highlights the broad utility of this mechanism as a general response device, even to nonoxidative genomic stress (Polyak et al., 1997).

Relevance for the organism

If, indeed, oxidants require a high degree of spatial ordering to confer signal fidelity, one might wonder why organisms did not evolve a more robust cytosolic antioxidant defense to completely suppress stray redox signals and minimize oxidant stress. One possible answer may be that an excessively high level of cytosolic antioxidants would be expected to dampen local redox signals. A second answer may lie in the speculation that oxidative stress pathways may have evolved before localized oxidant signaling, meaning that pathways related to the latter had to be retrofit into an organism that already used oxidant production and sensing in its alarm system. As mentioned earlier, in *S. cerevisiae* we find enhanced mitochondrial oxidant production after heat shock, leading to the activation of cytosolic reporters and transactivation of stress response genes;

therefore, exuberant scavenging of cytosolic oxidants may gainsay what, in this case, would be a protective stress response.

Both answers reveal the redox tightrope the cell is required to walk to spatially discriminate homeostatic from stress signaling. This issue becomes particularly vexing in regard to the mitochondrion, which, despite its ability to confine its oxidative effects locally, can alternatively flood the cell with oxidants and damage itself in the process, functioning as a principal loudspeaker for sounding oxidant stress alarms. This scenario highlights the exquisite control required for both oxidant production and its escape into the cytosol. The consequence of losing such control would appear to be inappropriate stress responses, such as unscheduled cell cycle arrest or apoptosis, or insensitivity to real stress with failure to activate these processes. Not surprisingly, human states that reflect these same cellular signaling defects result in either degenerative or neoplastic diseases that arise in the context of either excessive oxidative stress or insensitivity to such stress.

The ubiquity of SOD in aerobic cells indeed reflects the dire consequences of poor oxidant regulation. The cellular strategy of subsequently adopting these oxidants for signaling purposes appears to have required the evolution of spatial control, incorporation into other general signaling devices, and the preservation of a global oxidative distress pathway. When Emperor Joseph II complained about the commissioned opera *The Abduction from the Seraglio* that there were "too many notes, my dear Mozart," Mozart is said to have responded: "(There are) exactly the right number, your Majesty." This comment appears to apply to reactive oxidants as well, with the further caveat that they should be in exactly the right places.

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