

The general case for redox control of wound repair

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The orthodox view has been that reactive oxygen species are primarily damaging to cells. There is general agreement that while high (3%) doses of H₂O₂ may serve as a clinical disinfectant, its overall effect on healing is not positive. Current work shows that at very low concentrations, reactive oxygen species may regulate cellular signaling pathways by redox-dependent mechanisms. Recent discoveries show that almost all cells of the wound microenvironment contain specialized enzymes that utilize O₂ to generate reactive oxygen species. Numerous aspects of wound healing are subject to redox control. An understanding of how endogenous reactive oxygen species are generated in wound-related cells may influence the healing process and could result in new redox-based therapeutic strategies. Current results with growth factor therapy of wounds have not met clinical expectations. Many of these growth factors, such as platelet-derived growth factor, rely on reactive oxygen species for functioning. Redox-based strategies may serve as effective adjuncts to jump-start healing of chronic wounds. The understanding of wound-site redox biology is also likely to provide novel insights into the fundamental mechanisms that would help to optimize conditions for oxygen therapy. While a window of therapeutic opportunity seems to exist under conditions of low concentrations of reactive oxygen species, high levels may complicate regeneration and remodeling of nascent tissue. **(WOUND REP REG 2003;11:431-438)**

The orthodox view has been that the primary role of oxidants in biology is to incite oxidative damage. High concentrations of H₂O₂ (3%) are used clinically for wound disinfection. There is general agreement that at these doses H₂O₂ may not be beneficial for overall healing. This agreement is in line with the biochemistry literature, which unambiguously reports that such high doses of H₂O₂ are potentially toxic. Recent work shows that at concentrations much below that required to cause oxidative damage, oxidants may regulate intracellular signal transduction pathways by redox-dependent mechanisms.¹⁻⁹ Numerous

AP	Activator protein
FGF-2	Fibroblast growth factor-2
IGF	Insulin-like growth factor
MCP-1	Monocyte chemoattractant protein-1
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
PDGF	Platelet-derived growth factor
PI3K	Phosphatidyl inositol 3 kinase
RANTES	Regulated upon activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
TF	Tissue factor
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells

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proteins with apparent redox-sensing activity have been described. Electron flow through side-chain functional CH₂-SH groups of conserved cysteinyl residues represents a major mechanism for redox-sensing.⁶ Oxidants are now studied as second messenger molecules.^{10,11} This makes room for a new paradigm in the biology of wound healing.¹²

DELIBERATE PRODUCTION OF REACTIVE OXYGEN SPECIES BY WOUND RELATED CELLS

Classically, reactive oxygen species (ROS) have been extensively studied as damaging by-products accidentally “leaked” from the mitochondrial respiratory chain.¹³ In 1972 the Chance laboratory estimated that at least 1% of total oxygen uptake during state 4 respiration contributes to H₂O₂ formation.¹⁴ Later that value was revised upward to read that 3–5% of total oxygen consumed by the mitochondria generates ROS under basal conditions.^{15,16} Multi-fold increases in mitochondrial ROS production and associated oxidative damage have been reported in numerous conditions.^{15,17,18}

The significance of Sbarra and Karnovsky’s 1959 discovery of leukocyte oxidase¹⁹ in phagocytes became apparent in the late 1970s, when the pioneering work of Babior linked the explosive production of superoxide ions (O₂^{•-}) by leukocyte oxidase to bacterial killing.²⁰

Phagocytic NADPH oxidase

Babior’s discovery²⁰ propelled the science of leukocyte oxidase in phagocytes and resulted in the renaming of the enzyme to NADPH oxidase. NADPH oxidase catalyzes the production of ROS by cells and has been extensively investigated in phagocytes (neutrophilic and eosinophilic granulocytes, monocytes, and macrophages). Exposure of these cells to any of a large number of stimuli activates a “respiratory burst” caused by an activation of the plasma membrane-bound NADPH oxidase $\text{NADP}^+ + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{O}_2^{\bullet-} + \text{H}^+$. The O₂^{•-} then rapidly dismutates to H₂O₂.

The oxidase itself is highly complex, consisting of four unique subunits and Rac2. In the resting cell, two of the subunits, p22^{phox} and gp91^{phox}, are located in the membrane, and the other two, p47^{phox} and p67^{phox}, are in the cytosol (Figure 1). The electron-carrying components of the oxidase are located in gp91^{phox}; the NADPH binding site is generally regarded to be in gp91^{phox} as well, but there is some evidence that it may be in p67^{phox}. The catalytic subunit, gp91^{phox}, is dormant in resting cells but becomes activated by assembly with cytosolic regulatory proteins. When the oxidase is activated, p47^{phox} is phosphorylated at specific sites and the cytosolic components plus Rac2 migrate to the membrane to assemble the active oxidase.²¹

These ROS are used by phagocytes to kill invading microorganisms, but they also cause significant “collateral damage” in the form of inflammation to nearby tissues. Consequently, their production must be tightly regulated to make sure that ROS are generated only when and where required. This control is executed by a sophisticated signal

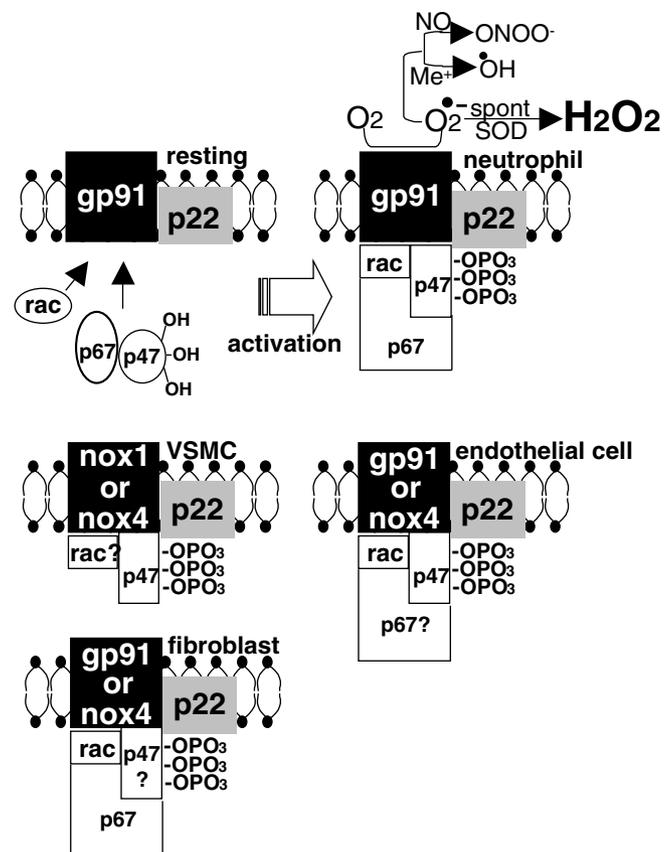


FIGURE 1. NADPH oxidases and their subunits in wound-related cells. Inducible activation and formation of H₂O₂, a membrane-permeable form of ROS more stable than other species.

transduction system characterized by the presence of multiple checkpoints.²² The phosphorylation of p47^{phox} is a major limiting step required for the assembly of the active NADPH oxidase enzyme complex.²³ Mutations in p47^{phox} are a cause of chronic granulomatous disease, an immune-deficient condition characterized by impaired healing response.^{24,25} Recently it has been observed that Rac2 mutation is another factor responsible for impaired human neutrophil NADPH oxidase function, low O₂^{•-} generation, and compromised wound healing.²⁶

Non-phagocytic NADPH oxidase

Inducible ROS generated in some nonphagocytic cells are implicated in mitogenic signaling.^{27,28} Increased ROS in Ras-transformed fibroblasts correlates with an increased mitogenic rate. Many types of cells also show increased production of ROS, and normal cells exposed to hydrogen peroxide or superoxide show increased proliferation and express growth-related genes. ROS are generated in response to growth factors.^{5,29} These lines of evidence clearly indicate that the process of deliberate generation of ROS is not restricted to phagocytic cells.

In 1999, the cloning of Mox1 marked a major step in establishing the presence of distinct NADPH oxidases in nonphagocytic cells.³⁰ Mox1 or p65Mox was described as encoding a homolog of the catalytic subunit of the superoxide-generating NADPH oxidase of phagocytes, gp91^{phox}. Mox1 messenger RNA is expressed in colon, prostate, uterus, and vascular smooth muscle, but not in peripheral blood leukocytes.³⁰ Later, Mox1 was renamed as Nox1, referring to NADPH oxidase.³¹

The Nox family of enzymes now consists of seven members in humans, with orthologs in mice, rats, *Drosophila*, and *c. elegans*. A family tree constructed by comparing the sequences of the gp91phox-homology domain reveals three subfamilies: a gp91phox-like group, a Duox group, and a more distant homolog consisting of a single member, Nox5. Size comparisons reveal that the gp91phox group and a small splice form of Nox 5 are similar in size to gp91phox, an approximately 65 kDa protein. Duox enzymes and a large splice variant of Nox5 are larger and contain additional predicted domains.³² Nox1-derived H₂O₂ in low concentrations functions as an intracellular signal that triggers a genetic program related to cell growth.³¹

Rac: regulator of inducible NADPH oxidase function

Rac is a member of the Rho family of small GTPases. Rac-GTP is a component of the membrane-assembled NADPH oxidase complex, and new evidence suggests that Rac-GTP interacts directly with the oxidase flavocytochrome, in addition to binding to the regulatory p67 subunit, to regulate electron transfer both independently and cooperatively from NADPH to molecular oxygen. Other recent studies suggest that Rac-GTP plays a dual role in NADPH oxidase activation and can initiate signaling pathways leading to translocation of cytosolic oxidase subunits in addition to functioning in the assembled enzyme complex.³³ Rac2 accounts for more than 96% of Rac in neutrophils.²⁶

The function of Rac GTPases is not restricted to phagocytic cells. The two predominant isoforms of Rac (Rac1 and Rac2) differ primarily in their C-termini, where Rac1 contains polybasic amino acid residues while Rac2 is less basic. Rac1 controls actin redistribution to membrane ruffles in fibroblasts and other cell types, as well as the activation of the NADPH oxidase in both phagocytes and nonphagocytic cells.^{27,28,34-37} Transient expression of a constitutively activated form of Ras in NIH3T3 cells induced a significant increase in intracellular ROS that was inhibited by the expression of a dominant negative allele of either Ras or Rac1.^{27,28} The ability of Rac to stimulate superoxide production relies upon its conversion

from the inactive GDP-bound form to the active GTP-bound form.

Recombinant adenoviral expression of a dominant negative Rac1 suppressed tissue damage in an in vivo model of mouse hepatic ischemia/reperfusion injury. This was also observed in mice deficient for the gp91^{phox} of phagocytic NADPH oxidase, suggesting that the Rac mutant inhibited ROS production by a Nox system rather than by one employing gp91^{phox}.³⁶ Thus, it appears that ROS production in nonphagocytic cells may involve the regulation of an NADPH oxidase-like enzyme by Rac GTPase.

Recently we have reported the first evidence supporting the hypothesis that active Rac1 gene transfer facilitates the healing of excisional dermal wounds in mice.³⁷ These findings are consistent with numerous reports in the literature showing that Rac1 regulates several aspects of cell biology that may be directly linked to the healing response. In a wide variety of cell types, Rac1 regulates signaling pathways that are critical for mitogenesis,²⁷ extracellular matrix,³⁴ chemotaxis,³⁸ actin remodeling,³⁹ cytoskeletal reorganization,⁴⁰ integrin complex formation,⁴¹ cell motility,⁴² and angiogenesis.^{37,43} While the involvement of ROS has been shown in many of these signaling cascades, Rac GTPases are likely to have oxidase-independent functions as well.

NADPH oxidase in response to wounding

Mutations in p47^{phox} are a cause of chronic granulomatous disease, an immune-deficient condition characterized by an impaired healing response.^{24,25} Rac2 mutation is another factor responsible for impaired human neutrophil NADPH oxidase function, low O₂⁻ generation, and compromised wound healing.²⁶ Yet another compelling line of evidence indicating a key role of neutrophil NADPH oxidase in wound healing comes from the study of burn wounds. Infection is a major cause of morbidity and mortality in patients after thermal injury. It has been determined in humans that NADPH oxidase activity of intact polymorphonuclear cells, measured as O₂⁻ generation or O₂ consumption, was decreased in the burn compared with healthy controls. This compromise in enzyme function has been directly linked to lower levels of both p47^{phox} and p67^{phox}.⁴⁴ The addition of purified or human recombinant p47^{phox} but not p67^{phox} corrected the diminished oxidase activity of cytosol from burn patients, showing that the level of p47^{phox} is the limiting factor.⁴⁴ In 1990, the work of Hunt and associates presented the first evidence leading to the hypothesis that wound response includes alterations in the assembly and function of the NADPH oxidase leading to enhanced O₂⁻ production by wound neutrophils.⁴⁵ Soon thereafter, it was independently verified that in response to trauma, p47^{phox} protein is significantly overexpressed

in polymorphonuclear cells, resulting in increased ROS production.⁴⁶

REDOX-REGULATED PROCESSES RELEVANT TO WOUND HEALING

Hemostasis represents one of the earliest responses in the healing process. Tissue factor (TF) initiates the extrinsic coagulation cascade leading to thrombin formation. Thrombin induces TF mRNA in vascular smooth muscle cells (VSMCs), thereby contributing to the prolonged procoagulant activity and enhanced thrombogenicity at sites of vascular injury. Recent findings show that NADPH oxidase is intimately involved in the redox-sensitive induction of TF mRNA expression and surface procoagulant activity by thrombin.⁴⁷ This response is mediated by NADPH oxidase-dependent activation of p38 MAP kinase and the PI 3-kinase/protein kinase B/Akt pathway. Given that active TF promotes thrombin formation, the NADPH oxidase may play a crucial role in perpetuating the thrombogenic cycle in the injured vessel wall.⁴⁷

Platelet aggregation and activation is another key player in reestablishing the hemostatic plug as well as in delivering facilitators of healing such as platelet-derived growth factor (PDGF). Platelets have the capacity to generate ROS⁴⁸ and at the wound-site colocalize with other ROS-generating cells such as white blood cells. Activated platelets up-regulate TF expression and this response involves ROS generation and a p22phox-containing NADPH oxidase in VSMC.⁴⁹ Collagen-induced platelet aggregation has been observed to be associated with production of H₂O₂, which was abolished by catalase, an enzyme that destroys H₂O₂.⁵⁰ Catalase dose-dependently inhibited thromboxane A₂ production, release of arachidonic acid from platelet membrane, and inositol 1,4,5-trisphosphate formation. In platelets, stimulated with high concentrations of collagen, catalase inhibited platelet aggregation, calcium mobilization, and inositol 1,4,5-trisphosphate production. This study suggested that collagen-induced platelet aggregation is associated with a burst of H₂O₂ that acts as a second messenger by stimulating the arachidonic acid metabolism and phospholipase C pathways.⁵⁰ More recently it has been observed that collagen activation induces NADPH oxidase-dependent O₂⁻ release in platelets, which in turn enhances availability of released ADP, resulting in increased platelet recruitment.⁵¹

PDGF represents a family of dimeric protein isoforms that stimulates growth, chemotaxis, and cell shape changes of various connective tissue cell types and other wound-related cells. PDGF is used clinically to effect wound healing. It should be noted that PDGF is dependent on H₂O₂ for its biological function.⁵² Becaplermin (0.01

percent Regranex[®] gel) is a homodimeric protein produced by recombinant DNA technology through the insertion of the gene for the B chain PDGF into the yeast *Saccharomyces cerevisiae*. The biological activity of becaplermin is similar to that of endogenous PDGF-BB, specifically, the promotion of chemotactic recruitment, and the proliferation of cells involved in wound repair. Engagement of PDGF with its receptor results in the generation of H₂O₂ in nonphagocytic cells by a PI3K- and Rac1-dependent pathway.⁵³ PDGF-stimulated O₂⁻ production modulates activation of transcription factor NF-κB and expression of monocyte chemoattractant protein-1 (MCP-1) in human aortic smooth muscle cells.⁵⁴

Neutrophils infiltrating into the wound site cleanse the wound of foreign particles and bacteria and are then extruded with the eschar or phagocytosed by macrophages. ROS generated by both neutrophils as well as macrophages play a central role in conferring resistance to wound infection.^{20,24–26,55,56} Blood coagulation, activated complement pathways, and activated parenchymal cells at the wound site generate numerous vasoactive mediators. Experiments studying the directed locomotion of mouse peritoneal neutrophils under agarose show that at low μM concentrations H₂O₂ induces neutrophil chemotaxis.⁵⁷ Overexpression of thioredoxin, a ROS decomposing cellular thiol, suppresses leukocyte recruitment induced by the murine chemokines KC/GROα, RANTES (regulated upon activation, normal T cell expressed and secreted), and MCP-1.⁵⁸ Monocytes are recruited to the wound site by specific chemoattractants such as fragmented extracellular matrix protein, transforming growth factor-β (TGF-β), MCP-1, and macrophage inflammatory protein (MIP). ROS induces MIP-1α, MIP-2, and MCP-1.^{59–61} ROS induces TGF-β expression as well as its activation by oxidatively displacing the latency-conferring peptide.⁶² In certain cell types, H₂O₂ is a requirement for TGF-β-induced cell signaling.⁶³ Monocytes adhere to specific proteins of the extracellular matrix by their integrin receptors. Such adhesion triggers the differentiation of monocytes to reparative macrophages and stimulates phagocytosis of microorganisms and fragments of extracellular matrix. H₂O₂ induces LFA-1-dependent neutrophil adherence and Mac-1-dependent macrophage adherence.⁶⁴ The ROS-decomposing antioxidant N-acetyl-L-cysteine suppresses constitutive expression of CD11a/LFA-1α protein in cells of myeloid lineage.⁶⁵ H₂O₂ modulates leukocyte adhesion molecule expression and leukocyte endothelial adhesion.⁶⁶ Adherence of monocytes to the extracellular matrix also induces the expression of monocyte colony-stimulating factor 1, a cytokine that supports monocyte and macrophage survival at the wound site. H₂O₂ is known to mediate the transcriptional induction of macrophage colony-stimulating factor.⁶⁷ Other

macrophage-derived cytokines expected at the wound site include tumor necrosis factor α and PDGF. As discussed above, PDGF function is subject to redox control at multiple levels. Tumor necrosis factor α biosynthesis has been shown to be ROS-inducible as well.^{68,69} Current evidence shows that several chemokines and their receptors respond to ROS. For example, the mRNA expression of the chemokine receptors CCR5 and CXCR4, and CCR2, CCR5, and CXCR4 mRNA expression is sensitive to low concentrations of H₂O₂. H₂O₂ increased cell migration (threefold) in response to MIP1.⁷⁰ Inducible interleukin-1 β and -6 expression is sensitive to ROS as well.⁶⁹ Cultured macrophages exhibit spreading in response to external stimuli that is relevant to in vivo morphologic changes of macrophages during extravasation, migration, and differentiation. ROS induce spreading of macrophages via the MAP kinase-SRE signaling pathways.⁷¹

Reepithelialization of wounds involves the formation of peripheral cytoplasmic actin filaments, which allow cell motility. ROS induce smooth muscle cell proliferation and migration.⁷² Specifically, H₂O₂ has been shown to induce pro-matrix metalloproteinase (MMP)-2 activation and cell motility.⁷³ The fibrinolytic mechanism that comprises the activation of plasminogen into plasmin prevents excessive fibrin accumulation by promoting local dissolution of thrombi and promoting wound healing by reestablishment of blood flow.⁷⁴ ¹O₂ converts fibrin to a form that stimulates the activation of plasminogen (bound to oxidized fibrin) by pro-urokinase and that of pro-urokinase by plasmin. The oxidative modification of fibrin by ¹O₂ is specific and favors subsequent fibrinolysis.⁷⁵ The degradation of extracellular matrix, required to allow motile wound-related cells to migrate, depends on the production of collagenase by epidermal cells as well as on fibrinolysis. Plasminogen activator also activates collagenase (MMP-1) and therefore facilitates degradation of extracellular matrix proteins, thus allowing wound cells to migrate. H₂O₂ has been identified to mediate AP-1-dependent induction of MMP-1.⁷⁶ One to two days after injury, epidermal cells at the wound margin begin to proliferate behind the actively migrating cells. Epidermal growth factor, transforming growth factor- α , and keratinocyte growth factor are thought to support this process. H₂O₂ plays a central role in triggering epidermal growth factor receptor phosphorylation and signaling.^{77,78} ROS are also known to induce transforming growth factor- α in fibroblasts.⁷⁹

The wound site starts to be occupied by granulation tissue approximately 4 days after injury. New capillaries primarily contribute to the granular appearance of the tissue. Macrophages, fibroblasts, and blood vessels are major components of the granulation tissue. Guided by

products contributed by macrophages, wound angiogenesis speeds up. Basic fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) are two key facilitators of wound angiogenesis. Other growth factors such as PDGF, TGF- β , and insulin-like growth factor-1 (IGF-1) presumably prepare the fibroblasts to participate in the formation of granulation tissue. The redox control of PDGF and TGF- β has been discussed in an earlier section of this article. IGF-1, known for its role in reepithelialization as well as granulation tissue formation, is inducible by ROS.⁸⁰ Furthermore, it has been shown that an oxidizing environment favors IGF-1 and IGF-2 function.⁸¹

H₂O₂ enhances the affinity of FGF-2 for its receptor.⁸² In addition, ROS induces FGF-2 expression.^{83,84} H₂O₂ induces VEGF expression in wound-related cells.^{12,37,85,86} We observed that H₂O₂ potently induces VEGF expression in human keratinocytes. Deletion-mutant studies with a VEGF promoter construct revealed that a GC-rich sequence from bp -194 to -50 of the VEGF promoter is responsible for this H₂O₂ response. It was established that at μ M concentrations oxidant induces VEGF expression and that oxidant-induced VEGF expression is independent of hypoxia-inducible factor-1 and dependent on Sp1 activation.³⁷ Among many known growth factors, VEGF is believed to be the most prevalent, efficacious, and long-term signal that is known to stimulate angiogenesis in wounds.⁸⁷ Recently it has been shown that Nox1-derived ROS is a potent trigger of the angiogenic switch, increasing vascularity and inducing molecular markers of angiogenesis. VEGF mRNA becomes markedly up-regulated by Nox1, and VEGF receptors (VEGFR1 and VEGFR2) are highly induced in vascular cells in Nox1-expressing tumors. Nox1 also induces MMP activity, another marker of the angiogenic switch. Nox1 induction of VEGF is eliminated by coexpression of catalase, indicating that hydrogen peroxide signals for part of the switch to the angiogenic phenotype.⁸⁸ H₂O₂-induced tubular morphogenesis (angiogenesis) of human microvascular endothelial cells is mediated by the redox-sensitive transcription factor NF- κ B.^{7,89} The various mechanisms involved in the redox regulation of angiogenesis have been recently reviewed.⁹⁰

A provisional matrix supports the formation of granulation tissue by providing a scaffold for tissue repair. This matrix is made up of structural molecules that are primarily contributed by the fibroblasts. Next, a cross-linked collagen matrix replaces the provisional matrix. Noncytolytic doses of H₂O₂ induce collagen I, III, and IV and TGF- β 1 mRNA.⁹¹⁻⁹³ H₂O₂ also supports collagen cross-linking.⁹⁴ Not only ROS but ambient molecular O₂ is also known to support collagen hydroxyproline formation.⁹⁵ Lysyl oxidase, an enzyme secreted by VSMCs, initiates the covalent cross-linking of polypeptide

chains within collagen and elastin. It has been established that purified lysyl oxidase strongly induces directional migration of VSMC in an H_2O_2 -dependent manner.⁹⁶ These processes are intricately related to extracellular matrix reorganization.

Wound contraction is an event that continues throughout the healing process. In murine models of dermal wound healing, wound contraction plays a major role in the early phase of healing. It has been recently shown that O_2 and ROS may trigger the differentiation of fibroblasts to myofibroblasts, a key mediator of the contractile process.⁶²

CONCLUSIONS

Taken together, it is clear that numerous aspects of wound healing are subject to redox control. Thus, development of a thorough understanding of how endogenous ROS generated in wound-related cells may influence the healing process becomes critically important. Such an understanding could result in novel redox-based strategies to treat wounds. Current results with growth factor therapy of wounds do not meet expectations. Many of these growth factors, like PDGF, rely on ROS for functioning. Thus, redox-based strategies may serve as effective adjuncts to jump-start the healing of a chronic wound. Hypoxia being a characteristic feature of most problem wounds, it is reasonable to assume that correction of wound pO_2 may facilitate generation of endogenous ROS by NADPH oxidases in wound-related phagocytic and nonphagocytic cells. Gas delivery⁹⁷ and molecular³⁷ approaches to facilitate functioning of NADPH oxidases in wound-related cells could prove to be productive. Therapeutic modalities relying on up-regulating ROS generation in the wound microenvironment will have to be met with caution. While a window of opportunity seems to exist under conditions of low concentrations of ROS, high levels of ROS clearly have the potential to complicate regeneration and remodeling of nascent tissue.

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